ENDOTHELIAL INJURY IN CARDIAC TRANSPLANTATION:
THE ROLE OF ENDOTHELIN ANTAGONISM AND PROTEIN KINASES

by

Danny Ramzy, MD

A thesis submitted in conformity with
the requirements for the degree of
Doctor of Philosophy
Graduate Department of the Institute of Medical Science,
University of Toronto,

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ENDOTHELIAL INJURY IN CARDIAC TRANSPLANTATION: THE ROLE OF ENDOTHELIN ANTAGONISM AND PROTEIN KINASES

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Doctor of Philosophy
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2008

ABSTRACT

BACKGROUND: Endothelial dysfunction is a principal player in the development of allograft vasculopathy and allograft failure. The hallmark of endothelial dysfunction is impaired nitric oxide bioavailability. Recent evidence implicates endothelin-1 as an integral component of endothelial dysfunction. Immunosuppressive drugs have also been associated with the development of graft vasculopathy. We speculated that endothelin-1 results in endothelial dysfunction by impairing nitric oxide homeostasis and is a player in hypoxia and reperfusion induced vasomotor injury. In addition, we hypothesized that endothelin-1 antagonism with bosentan will limit hypoxia and reperfusion injury and prevent immunosuppressive drug injury. METHODS: We utilized human saphenous vein endothelial cells to evaluate the effects of endothelin-1, hypoxia and reperfusion on endothelial function, protein kinase modulation and cell survival. We also employed a rodent model of chronic drug therapy to assess the effect of cyclosporine and rapamycin treatment on vasomotor function. We investigated the role of nitric oxide augmentation and bosentan in preventing hypoxia and reperfusion injury and in limiting immunosuppressive drug induced vasomotor dysfunction. RESULTS: Elevated endothelin-1 levels resulted in
impaired nitric oxide release and endothelial function. The effects of endothelin-1 as well as hypoxia and reperfusion were mediated by altered protein kinase B and protein kinase C activity resulting in endothelial dysfunction. We revealed that endothelin-1 is a key player in hypoxia and reperfusion induced endothelial injury. The immunosuppressive drug cyclosporine induced vasomotor dysfunction while rapamycin preserved vessel homeostasis. Vasomotor dysfunction was characterized by impaired nitric oxide and endothelin-1 homeostasis. Bosentan limited the deleterious effects of endothelin-1, hypoxic injury, reperfusion injury and cyclosporine induced vasomotor impairment.

**CONCLUSIONS:** Our study revealed that endothelin-1 exposure as well as hypoxia and reperfusion results in endothelial dysfunction by altering specific protein kinase C isoform activities and inhibiting protein kinase B. Cyclosporine induced vasomotor dysfunction was mediated by altered nitric oxide and endothelin-1 homeostasis while rapamycin was endothelial protective. Bosentan proved to be an effective therapy at preventing endothelin-1, hypoxia and reperfusion and cyclosporine induced endothelial dysfunction. Protein kinase C modulation as well as bosentan may prove to be NOVEL therapies to prevent endothelial injury during cardiac transplantation.
DEDICATION

I dedicate my thesis in loving memory to my father Alphonse Talatinian. His support and sacrifices helped me pursue my goals. I will always remember his help, guidance and love he gave me throughout my life.

I would also like to dedicate my thesis to my family. My loving mother GiGi Ramzy, whose sacrifices and tireless support of my interests and goals made it possible for me to attain my dreams. I will forever be grateful for her love and support.

To my sister Kathy Ramzy I am greatly appreciative of her love, support and sacrifices throughout my studies. I would also like to dedicate my thesis to my nephew Zane Ramzy and brother-in-law Patrick McConnell.
ACKNOWLEDGEMENT

The completion of this thesis would not be possible without the collaboration of my colleagues within the Division of Cardiovascular Surgery at the University of Toronto.

I would like to thank my mentor and supervisor, Dr. Vivek Rao, for his dedication, guidance, and encouragement. His tireless support allowed me to complete my work and acquire the necessary skills and knowledge to complete my thesis. I am and will be forever appreciative to him for introducing me to the practice of academic cardiac surgery as well as the world of clinical cardiac transplantation.

I would also like to thank my thesis committee members, Dr. Catharine Whiteside and Dr. Terrence Yau. Their dedication, support and advice were instrumental towards the completion of my thesis.

I would also like to acknowledge the help of Laura Tumiai and Ning Xu in the completion of this study.

Lastly, I would like to thank the Thoracic Surgery Foundation for Research and Education, Tailored Advanced Collaborative Training in Cardiovascular Science, and the Physician Services Incorporated Foundation for their funding support of this work.
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<td>Ach</td>
<td>Acetylcholine</td>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
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<td>AKAP79</td>
<td>A Kinase-Anchoring Protein 79</td>
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<td>AP-1</td>
<td>Activator protein-1</td>
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<tr>
<td>Apocynin</td>
<td>4-hydroxy-3-methoxy-acetiphenone</td>
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<td>ApoE</td>
<td>Apoprotein-E</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BH₄</td>
<td>Tetrahydrobiopterin</td>
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<tr>
<td>BHT</td>
<td>Butylated hydroxy toluene</td>
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<td>BOOP</td>
<td>Bronchiolitis obliterans with organizing pneumonia</td>
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<td>BOS</td>
<td>Bosentan</td>
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<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
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<tr>
<td>Cal</td>
<td>Calphostin C</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>Caspase</td>
<td>Cysteiny1-aspartic-acid-proteases</td>
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<td>CAV</td>
<td>Cardiac allograft vasculopathy</td>
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<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
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<td>Chel</td>
<td>Chelerythrine</td>
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<tr>
<td>CK</td>
<td>Creatine kinase</td>
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<tr>
<td>%Cmax</td>
<td>Maximum increase in tension from baseline</td>
</tr>
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<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CRE</td>
<td>cAMP-responsive element</td>
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<td>CREB</td>
<td>CRE-binding protein</td>
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<td>Cyclosporine A</td>
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<td>DAG</td>
<td>Diacylglycerol</td>
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<td>Asp-Glu-Val-Asp-Rhodamine 110</td>
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<td>DI</td>
<td>Rottlerin</td>
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<tr>
<td>DISC</td>
<td>Death-inducing signaling complex</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DSE</td>
<td>Dobutamine stress echocardiography</td>
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<tr>
<td>ED₅₀</td>
<td>Concentration required to achieve half-maximum vasorelaxation</td>
</tr>
<tr>
<td>Eⅵ</td>
<td>Myristoylated PKCε inhibitory peptide</td>
</tr>
<tr>
<td>EIAB</td>
<td>Enzyme immunoassay buffer</td>
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<tr>
<td>Emax%</td>
<td>% maximal relaxation from phenylephrine-induced vasoconstriction</td>
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<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
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<td>ET-1</td>
<td>Endothelin-1</td>
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<td>ETₐ A</td>
<td>ET-1 receptor A</td>
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<tr>
<td>ETₐ B</td>
<td>ET-1 receptor B</td>
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<tr>
<td>FADD</td>
<td>FAS death domain adaptor</td>
</tr>
<tr>
<td>FASL</td>
<td>FAS ligand</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>FKBP</td>
<td>FK-binding proteins</td>
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<td>FOS</td>
<td>Forskolin</td>
</tr>
<tr>
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<td>Forkhead</td>
</tr>
<tr>
<td>H₂DCFDA</td>
<td>Dichlorodihydroflurescein diacetate</td>
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<td>H89</td>
<td>N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (\cdot) 2HCl</td>
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<td>Hcy</td>
<td>Homocysteinemia</td>
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<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
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<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid</td>
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<td>Hyperhomocysteinemia</td>
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<td>H/R</td>
<td>Hypoxia and reperfusion</td>
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<tr>
<td>HSVEC</td>
<td>Human saphenous vein endothelial cell</td>
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<td>ICAM</td>
<td>Intracellular adhesion molecule</td>
</tr>
<tr>
<td>IEL</td>
<td>Intenal elastic lamina</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>INF-γ</td>
<td>Interferon-γ</td>
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<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<td>IP3</td>
<td>Inositol-1,4,5 triphosphate</td>
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<td>Ischemic preconditioning</td>
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<td>Ischemia and reperfusion</td>
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<tr>
<td>IVUS</td>
<td>Intravascular ultrasound</td>
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<tr>
<td>LAD</td>
<td>Left anterior descending coronary artery</td>
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<td>LDL</td>
<td>Low-density lipoprotein</td>
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<td>L-NAME</td>
<td>L-arginine-methyl-ester</td>
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<tr>
<td>LV</td>
<td>Left ventricular</td>
</tr>
<tr>
<td>M/C</td>
<td>Membrane-to-cytosolic</td>
</tr>
<tr>
<td>MDC</td>
<td>Monodansylcadaverine</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>mtNOS</td>
<td>Mitochondrial nitric oxide synthase</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target or rapamycin</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NF-1</td>
<td>CAAT-binding factor</td>
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<td>NFAT</td>
<td>Nuclear factor of activated T-cell</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide radical</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>Oxygen radical</td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Preconditioning</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterases</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phosphoinositol dependent kinase-1</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-OH kinase</td>
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<td>Abbreviation</td>
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</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<tr>
<td>PKIP</td>
<td>Protein kinase peptide inhibitor</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol-4,5 disphosphate</td>
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<tr>
<td>PIP3</td>
<td>Phosphatidylinositol-1,4,5 triphosphate</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethlysulphonylfluoride</td>
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<tr>
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<td>Very low density lipoprotein</td>
</tr>
<tr>
<td>ZI</td>
<td>Myristoylated PKC inhibitor</td>
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Figure 2. Maximal intimal thickness one year after cardiac transplantation. Pravastatin significantly attenuated intimal proliferation during the first year following transplantation.

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Figure 3. Mechanism of preconditioning. This figure demonstrates the central role that PKC can play in preconditioning. PKC can be activated by preconditioning triggers and be responsible for signal transduction that eventually results in an end-effector effect.

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Figure 4. Two step reaction of nitric oxide formation. Step1, L-arginine is converted to $N^G$-hydroxyl-L-arginine. Step2, $N^G$-hydroxyl-L-arginine is converted to L-citrulline and nitric oxide.

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Figure 5. Classical PKC Activation. Classical PKC activation occurs by binding of a ligand to its G-protein coupled receptor leading to PLC activation and the formation of IP$_3$ and DAG. IP$_3$ increases cytosolic calcium concentrations. Both calcium and DAG lead to PKC activation.

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Figure 6. Schematic representation of PKC structure. PKC is composed of a regulatory domain and a catalytic domain. The regulatory domain determines DAG and or PS binding. The regulatory domain also contains the autoinhibitory pseudosubstrate. The catalytic domain contains the active site. When PKC is activated the active site performs the kinase function.

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Figure 7. Schematic representation of PKA structure. PKA is composed of two subunits: the regulatory subunit and the catalytic subunit. The regulatory subunit allows for dimerization and inhibition of the catalytic subunit. The catalytic subunit confers the kinase function and does not require activation for activity. The catalytic subunit is constitutively active in the monomeric form and therefore dimerization is the only method of inhibition.

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Figure 8. Schematic representation of PKA activity. PKA is in a tetrameric inactive state. Upon activation of cAMP, two molecules of cAMP bind to the regulatory subunit and decrease affinity of the regulatory subunit for cAMP. Two more cAMP molecules are required to disassociate the catalytic (CS) subunits which do not require activation for kinase activity.

Figure 9. Schematic representation of ET-1 structure. ET-1 is a 21 amino acid protein with two disulfide bonds between cysteine 3 and 11 as well as cysteine 1 and 15. These bonds are important for ET-1 activity. The terminal amino acid is tryptophan which is also crucial for activity.

Figure 10. A, Western Blot for plasma calcium-ATPase demonstrated no protein presence in the cytosolic fraction indicating no membrane contamination of the cytosolic fraction. B, Western blot analysis for β-actin showing no protein presence in the membrane fraction revealing no cytosolic contamination of the membrane fraction. Legend: M: membrane fraction; C: cytosolic fraction.

Figure 11. Dose-response effect of ET-1 treatment on HSVEC NO production. ET-1 resulted in a concentration-dependent decrease in NO production in HSVEC at concentrations above 1nM. Treatment with 1nM concentration of ET-1 significantly increased NO production. However, exposure to ET-1 concentrations below 1nM had no effect on NO production. Legend: Con: Control; ET: Endothelin-1; NO: Nitric oxide; HSVEC: Human saphenous vein endothelial cell.

Figure 12. NO production in HSVEC following treatment for 30 minutes and 24 hours. The presence of ET-1 significantly reduced NO production following 30 minutes with a greater inhibition after 24 hours. Exposure to the PKC inhibitors calphostin C and chelerythrine significantly lowered NO release from HSVECs. However, co-incubation of ET-1 with PKC inhibitors demonstrated no added reduction in NO levels. HSVEC exposed to PMA demonstrated an increased NO production compared to control and significantly abrogated ET-1 induced effects. BOS treatment was effective at blocking ET-1 effects at both 30 minutes and 24 hours. Legend: NO: Nitric Oxide; HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan.

Figure 13. NO production in HSVEC following treatment for 24 hours. The presence of ET-1 significantly reduced NO production following 24 hours. Exposure to
DI did not effect NO production while ZI and EI reduced NO release. Co-incubation of ET-1 with ZI or EI further decreased NO levels. DI exposure limited ET-1 effects on NO impairment. Legend: NO: Nitric oxide; HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; ZI: PKC\(\lambda\) inhibitor, DI: Rottlerin; EI: PKCe V1-2.

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Figure 14. NO production in HSVEC following treatment for 30 minutes and 24 hours. The presence of ET-1 significantly reduced NO production following 30 minutes with a greater inhibition after 24 hours. Exposure to the PKA inhibitor H89 did not alter NO production. HSVEC exposed to the PKA agonist FOS demonstrated no change in NO production compare to control. Modulation of PKA activity did not alter ET-1 induced impairment of NO homeostasis. Legend: NO: Nitric Oxide; HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; FOS: Forskolin (PKA agonist).

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Figure 15. NO production in HSVEC following treatment for 30 minutes and 24 hours. The presence of ET-1 significantly reduced NO production following 30 minutes with a greater inhibition after 24 hours. Exposure to the Akt/PKB inhibitor SH5 reduced NO production. Co-incubation of SH5 with ET-1 demonstrated a significant additive reduction in total nitrite levels. Legend: NO: Nitric Oxide; HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1.

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Figure 16. ROS production in HSVEC following treatment for 24 hours. The presence of ET-1 significantly increases ROS production. Exposure to BOS attenuates ET-1 induced effects. PKC inhibition decreases ROS production while PMA increased ROS release. SH5 treatment results in free radical generation. PMA and SH5 aggravated ET-1 induced ROS release. PKA modulation had no effect on ROS production. Legend: ROS: Reactive oxygen species; HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; BOS: Bosentan; Cal: Calphostin C; Chel: Chelerythrine; FOS: Forskolin (PKA agonist).

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Figure 17. ROS production in HSVEC following treatment for 24 hours. ZI and PKCε V1-2 exposure inhibited ROS production while DI did not demonstrate any difference compared to control. Co-incubation of ET-1 with PKCε V1-2 or DI resulted in a significant reduction in intracellular ROS production compared to ET-1 alone. Co-incubation with ZI did not lead to any significant changes compared to ET-1. Legend: ROS: Reactive oxygen species; HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; ZI: PKCλ inhibitor, DI: Rottlerin; EI: PKCε V1-2.

Figure 18. ROS production in HSVEC following treatment for 24 hours. In control cells sepiapterin (NOS cofactor), L-NAME (NOS inhibitor), apocynin (NADPH oxidase inhibitor) and tiron (SOD anion) did not significantly lower ROS production. Tempol (SOD mimetic) decreased ROS production. The use of sepiapterin, L-NAME, apocynin and tempol reduced ET-1 derived ROS, while tiron had no significant effect on ET-1 induced ROS production. Legend: ROS: Reactive oxygen species; HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; H2O2: hydrogen peroxide; Sep: Sepiapterin; TI: Tiron; TE: Tempol.

Figure 19. A, Western Blot for PKCα. PKCα was expressed solely on the membrane fraction. No significant differences were seen between groups. B, Western Blot for PKCι. PKCι was expressed solely on the cytosolic fraction. No significant differences were seen between groups. Legend: Control; ET: Endothelin-1; BOS: Bosentan; +’ve: Positive control; M: membrane fraction; C: cytosolic fraction.

Figure 20. PKCδ Translocation. A. Western Blots for PKCδ protein expression demonstrating significant differences between groups. B, Thirty minute exposure to ET-1 significantly translocated PKCδ to the membrane compared to control. Treatment with PKC inhibitors significantly reduced the M/C ratio suggesting an increased cytosolic expression of PKCδ, whereas PMA resulted in a significantly higher M/C ratio in the control group and not in the ET-1 exposed cells. Bosentan treatment abrogated ET-1 induced translocation. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); +’ve: Positive control.

Figure 21. PKCδ Translocation. A. Western Blots for PKCδ protein expression demonstrating significant differences between groups. B. 24 hour exposure to ET-1 caused PKCδ translocation. PKC inhibition for 24 hour reduced while PMA increased the M/C ratio only in the control group. Bosentan exposure attenuated ET-1 induced effects. Legend: Con: Control; ET:
Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); +’ve: Positive control.

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Figure 22. PKCε Translocation. A. Western Blots for PKCε protein expression demonstrating significant differences between groups. B, PKCε was significantly translocated to the membrane following ET-1 treatment compared to control. Treatment with PKC inhibitors significantly reduced the M/C ratio in ET-1 exposed cells while having no effect on the control group. PMA resulted in a significantly higher M/C ratio in the control group and not in the ET-1 exposed cells. Bosentan exposure attenuated ET-1 induced translocation. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); +’ve: Positive control.

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Figure 23. PKCε Translocation. A. Western Blots for PKCε protein expression demonstrating significant differences between groups. B, 24 hour exposure to ET-1 caused PKCε translocation. PKC inhibition for 24 hour lowered the M/C ratio only in the ET-1 group while PMA increased the M/C ratio only in the control group. HSVEC exposed to Bosentan blocked ET-1 induced effects. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); +’ve: Positive control.

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Figure 24. PKCλ Translocation. A. Western Blots for PKCλ protein expression demonstrating significant differences between groups. B, The presence of ET-1 for 30 minutes resulted in a significant lowering of the PKCλ M/C ratio. Treatment with PKC inhibitors significantly reduced the M/C ratio in the control group while having no effect on the ET-1 treated cells. PMA resulted in a significantly higher M/C ratio in both the control and ET-1 groups. BOS exposure in addition to attenuating ET-1 induced effects also resulted in a significantly higher translocation of PKCλ. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); +’ve: Positive control.

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Figure 25. PKCλ Translocation. A. Western Blots for PKCλ protein expression demonstrating significant differences between groups. B, Exposure of HSVEC to ET-1 for 24 hours caused a significant lowering of the PKCλ M/C ratio. PKC inhibition for 24 hours lowered the M/C ratio while PMA increased the M/C ratio. HSVEC exposed to BOS blocked ET-1 induced effects while treatment of cells with BOS alone also resulted in an increased PKCλ M/C ratio. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin
Figure 26. HSVEC PKC activity. ET-1 caused a significant decrease in PKC activity following 30 minute exposure with a further reduction after 24 hours. Exposure to PKC inhibitors (Cal and Chel) resulted in a lowering of HSVEC PKC activity while PMA increased activity. BOS exposure blocked ET-1 reduction in PKC activity and increased PKC activity compared to control. Legend: HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chelerythrine; BOS: Bosentan.

Figure 27. HSVEC PKC activity. Akt/PKB inhibition with SH5 slightly reduced PKC activity following 30 minutes with no further reduction after 24 hours. Co-incubation with ET-1 resulted in a significant reduction compared to control but not compared to ET-1 at 30 minutes and 24 hours. PKA modulation with either FOS or H89 did not result in any significant changes in PKC activity and did not alter the ET-1 induced reduction in PKC activity. Legend: HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; FOS: Forskolin (PKA agonist).

Figure 28. HSVEC PKC activity. ZI specifically inhibited PKCλ in our endothelial cells. ET-1 exposed cells demonstrated a significant reduction in PKCλ activity compared to control but was significantly higher than PKCλ activity with ZI (F=53.81, p<0.0001). PMA resulted in significant activation of PKCλ. Legend: HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; ZI: PKCλ inhibitor; Cal: Calphostin C; Chel Chelerythrine.

Figure 29. HSVEC PKA activity. ET-1 exposure regardless of duration did not alter PKA activation compared to control. Modulation of PKC activity also failed to change cellular PKA activation. HSVEC treated with BOS did not modify PKA activity. Legend: HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan.

Figure 30. HSVEC PKA activity. Modulation of PKB activity failed to change cellular PKA activation. The PKA agonist FOS led to PKA activation while therapy with the PKA antagonist H89 resulted in decreased activity compared to all other groups. There was a time-dependent decrease in activity following H89 treatment and a time-dependent increase following FOS exposure. Legend: HSVEC: Human saphenous vein endothelial cell; Con: Control; ET:
Endothelin-1; FOS: Forskolin (PKA agonist).

Figure 31. Akt Phosphorylation. A. i-iii) Western blot for total and phospho-Akt expression demonstrated significant differences between groups at 30 minutes. B i) Quantitative Western blot analysis of phospho-Akt/Total Akt ratio. ET-1 reduces both serine and threonine phosphorylation compared to control. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; +’ve: Positive control.

Figure 32. Akt Phosphorylation. A. i-iii) Western blot for total and phospho-Akt expression demonstrated significant differences between groups at 24 hours. B i) Quantitative Western Blot analysis of phospho-Akt/Total Akt ratio. ET-1 reduces both serine and threonine phosphorylation compared to control. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; +’ve: Positive control.

Figure 33. Caveolin-1 protein expression. A. Western blot for caveolin-1 expression demonstrated no differences between groups following 30 minutes. B. Western blot for caveolin-1 expression demonstrated no differences between groups after 24 hour exposure to treatment. C. Western blot for β-actin expression demonstrated no significant differences between groups. Legend: +’ve: iNOS positive control. Con: Control; ET: endothelin-1; BOS: Bosentan; Cal: Calphostin C; Chel: Chelerythrine; FOS: Forskolin (PKA agonist).

Figure 34. iNOS protein expression. A. Western blot for iNOS expression demonstrated no induction following 30 minutes. B. Western blot for iNOS expression demonstrated no induction following 24 hours of exposure to treatment. Legend: MW: Molecular Weight; +’ve: iNOS positive control.

Figure 35. eNOS protein Expression. A. i-ii) Western blot for eNOS expression demonstrated no significant differences between groups at 30 minutes. iii) Western blot for β-actin expression demonstrated no significant differences. B i) Quantitative Western blot analysis of eNOS protein expression after 30 minutes of treatment demonstrated no significant differences between groups. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); +’ve: Positive control.
Figure 36. eNOS protein Expression. A. i-iii) Western blot for eNOS following 24-hour exposure demonstrated significant differences between groups. iv) Western blot for β-actin expression demonstrated no significant differences. B i) Quantitative Western blot analysis of eNOS protein expression after 24 hours of treatment demonstrated that ET-1 exposure downregulates eNOS protein expression compared to control and BOS abrogates the effects of ET-1. PKC inhibition with Cal or Chel decreased eNOS expression while the PKC agonist PMA increased expression. PKA and PKB modulation had no effect on eNOS expression. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); +‘ve: Positive control.

Figure 37. eNOS Localization. A. Western Blot depicting membrane and cytosolic eNOS protein expression. B. Exposure of HSVEC to ET-1 for 30 minutes caused a significant increase in the eNOS M/C ratio. PKC inhibition for 30 minutes with Cal or Chel increased the M/C ratio while the PKC agonist PMA decreased the M/C ratio. BOS exposure blocked ET-1 induced effects. SH5 increased ET-1 induced eNOS membrane sequestration. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); +‘ve: Positive control.

Figure 38. eNOS Localization. A. Western Blot depicting membrane and cytosolic eNOS protein expression. B. Exposure of HSVEC to ET-1 for 24 hours caused a significant increase in the eNOS M/C ratio. PKC inhibition for 24 hours increased the M/C ratio while PMA decreased the M/C ratio. BOS exposure blocked ET-1 effects. SH5 increased ET-1 induced eNOS membrane sequestration. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); +‘ve: Positive control.

Figure 39. Cell Viability. H/R decreased cell viability. ET-1 exposure further reduced cell viability. BOS therapy improved HSVEC tolerance to both H/R and ET-1 induced injury. Exposure to zvad, a caspase inhibitor, during hypoxia alone attenuated the effects of ET-1 and only reduced the effects of H/R when given during both hypoxia and reperfusion. Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; BOS: Bosentan; Hyp: Hypoxia; H/R: Hypoxia and reperfusion.

Figure 40. Cell Viability and PKC Modulation. PKC inhibition during hypoxia with Cal or Chel demonstrated a significant reduction in viability compared to control after 4 hours of reperfusion with a further decrease after 24 hours. PMA led to a decreased cell viability compared to control at both one and 24 hours of
reperfusion. Co-incubation of Cal, Chel or PMA with ET-1 led to a further impairment in cell viability compared to control, Cal, Chel or PMA. Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine.

Figure 41. Cell Viability and PKA/PKB Modulation. Inhibition of Akt/PKB with SH5 significantly reduced viability compared to control (4 and 24 hours) and ET-1 (24 hours). ET-1 co-incubated with SH5 for 4 hours impaired viability compared to SH5 alone, but by 24 hours there were no differences between these two groups. Altering PKA activity with FOS or H89 had no significant impact on H/R nor ET-1 induced injury. Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; FOS: Forskolin (PKA agonist).

Figure 42. Cell Viability and Treatment During Reperfusion. BOS given after hypoxia restored viability to near normoxic levels. BOS exposure during H/R maintained cell viability. Extreme reduction in viability was seen following ET-1 exposure during H/R. BOS was able to partially attenuate SH5 induced impairment and completely block the synergistic effect of ET-1 on SH5-induced injury. Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; BE: BOS+ET; Con/BOS: treatment with Bosentan (BOS) during reperfusion; ET/BOS: Hypoxic treatment with ET and treatment with BOS during reperfusion; ET/ET: ET treatment during hypoxia and reperfusion (H/R); BOS/BOS: BOS during both hypoxia and reperfusion; BE/BE: BE during both hypoxia and reperfusion.

Figure 43. Homogeneous Caspase Activity. Following hypoxia significant activation of homogeneous caspases (3, 6, 7) was observed. Reperfusion resulted in a time dependent increase in caspase activity. ET-1 exposure during hypoxia enhanced caspase activation following H/R. BOS therapy abrogated both H/R and ET-1 induced effects. The caspase inhibitor zvad attenuated caspase activation following H/R. Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; BOS: Bosentan; Hyp: Hypoxia; H/R: Hypoxia and reperfusion.

Figure 44. Homogeneous Caspase Activity. Hydrogen peroxide treatment during hypoxia increased caspase activation. When hydrogen peroxide was added during hypoxia and reperfusion further caspase activation occurred. Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; H2O2: Hydrogen peroxide; H/R: Hypoxia and reperfusion.
Figure 45. Homogeneous Caspase Activity. PKC inhibition resulted in greater caspase activation compared to control following each time point. PKC activation also resulted in greater caspase activation compared to control. Co-incubation with ET-1 resulted in an added increase in caspase activity. Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; Hyp: Hypoxia; H/R: Hypoxia and reperfusion.

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Figure 46. Homogeneous Caspase Activity. PKA modulation did not alter the HSVEC response to H/R or ET-1 induced caspase activation. Akt/PKB inhibition significantly increased caspase activation following H/R. Co-incubation of SH5 with ET-1 further increased caspase activation. Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; FOS: Forskolin (PKA antagonist); Hypoxia; H/R: Hypoxia and reperfusion.

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Figure 47. Homogeneous Caspase Activity. PKC\(\lambda\) inhibition during hypoxia significantly increased caspase activation compared to H/R alone while DI and EI significantly reduced caspase activity during all time points. ZI incubation during hypoxia and reperfusion showed increased caspase activity compared to control after one hour of reperfusion with no differences at 24 hours. DI and EI therapy during hypoxia significantly reduced ET-1 induced caspase activation following both hypoxia and reperfusion. Further protection against the development of apoptosis was seen when DI and EI were given during hypoxia and reperfusion. ET-1 co-incubation with ZI during hypoxia or H/R resulted in an additive increase in caspase activity. Legend: No inh: No inhibitor; ZI: PKC\(\lambda\) Inhibitor; DI: Rottlerin; EI: PKC\(\varepsilon\) inhibitor; Con: Control; ET: Endothelin-1; H/R: Hypoxia and reperfusion.

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Figure 48. Homogeneous Caspase Activity. Antioxidant (AO) therapy during hypoxia reduced caspase activation compared to No AO. Reperfusion with AO further decreased caspase activation. Greater reduction in caspase activity was observed with Sep (NOS cofactor), L-NAME (NOS inhibitor) and apocynin (NADPH oxidase inhibitor) compared to TE (SOD mimetic) and TI (SOD anion). Sep and L-NAME offered better protection versus apocynin. Legend: No AO: No antioxidants; Con: Control; Sep: sepiapterin; TE: Tempol; TI: Tiron; H/R: Hypoxia and reperfusion.

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Figure 49. Homogeneous Caspase Activity. Antioxidant (AO) therapy during hypoxia in the ET-1 group reduced caspase activation compared to No AO. Reperfusion with AO further decreased caspase activation. Greater reduction in caspase activity was observed with Sep (NOS cofactor), L-NAME (NOS inhibitor) and apocynin (NADPH oxidase inhibitor) compared to TE (SOD mimetic) and TI (SOD anion). Sep and L-NAME offered better protection
versus apocynin. Legend: No AO: No antioxidants; ET: Endothelin-1; Sep: sepiapterin; TE: Tempol; TI: Tiron; H/R: Hypoxia and reperfusion.

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Figure 50. Homogeneous Caspase Activity. Antioxidant (AO) therapy with BOS offered an approximate 10% decrease in apoptosis during reperfusion compared to BOS alone. Legend: No AO: No antioxidants; BOS: Bosentan; Sep: sepiapterin (NOS cofactor); TE: Tempol (SOD mimic); TI: Tiron (SOD anion); H/R: Hypoxia and reperfusion.

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Figure 51. Homogeneous Caspase Activity. Even in the presence of ET-1, antioxidant (AO) therapy with BOS resulted in an approximate 10% decrease in apoptosis during reperfusion compared to BOS alone. Legend: No AO: No antioxidants; BOS+ET: Bosentan+endothelin-1; Sep: sepiapterin (NOS cofactor); TE: Tempol (SOD mimic); TI: Tiron (SOD anion); H/R: Hypoxia and reperfusion.

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Figure 52. Homogeneous Caspase Activity. BOS therapy during hypoxia and reperfusion attenuated the effects of H/R, ET and SH5 on caspase activation. ET-1 treatment during both H and R enhanced caspase activity after H/R compared to all other treatment groups. Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; BOS: Bosentan; BE: BOS+ET; Con/BOS: treatment with BOS during reperfusion; ET/BOS: Hypoxia with ET and treatment with BOS during reperfusion; ET/ET: ET during hypoxia and reperfusion (H/R); BOS/BOS: BOS during H/R; BE/BE: BE during H/R.

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Figure 53. Caspase 8 Activity. Following hypoxia, a significant activation of caspase 8 was observed. Reperfusion resulted in a time dependent increase in caspase 8 activity. ET-1 exposure during hypoxia enhanced caspase 8 activation following H/R. BOS therapy abrogated both H/R and ET-1 induced activation. The caspase inhibitor zVAD attenuated caspase 8 activation following H/R. Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; BOS: Bosentan; Hyp: Hypoxia; H/R: Hypoxia and reperfusion.

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Figure 54. Caspase 8 Activity. PKC inhibition and activation resulted in greater caspase 8 activation following H/R than control. Co-incubation with ET-1 resulted in an added increase in caspase 8 activity. A reperfusion time dependent increase in caspase 8 activity was observed. Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; Hyp: Hypoxia; H/R: Hypoxia and reperfusion.

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Figure 55. Caspase 8 Activity. PKA modulation did not alter the response of HSVEC to H/R or ET-1 induced caspase 8 activation. Akt/PKB inhibition significantly increased caspase 8 activation following H/R. Co-incubation of SH5 with ET-1 further increased caspase 8 activation. Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; FOS: Forskolin (PKA agonist); Hypoxia; H/R: Hypoxia and reperfusion.

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Figure 56. Caspase 8 Activity. BOS therapy during hypoxia and reperfusion attenuated the effects of H/R, ET and SH5 on caspase 8 activation. ET-1 treatment during both H and R enhanced caspase 8 activity after H/R compared to all other treatment groups. Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; BOS: Bosentan; BE: BOS+ET; Con/BOS: treatment with BOS during reperfusion; ET/BOS: Hypoxia with ET and treatment with BOS during reperfusion; ET/ET: ET during hypoxia and reperfusion (H/R); BOS/BOS: BOS during H/R; BE/BE: BE during H/R.

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Figure 57. Caspase 9 Activity. Following hypoxia, a significant activation of caspase 9 was observed. Reperfusion resulted in a time dependent increase in caspase 9 activity. ET-1 exposure during hypoxia enhanced caspase 9 activation following H/R. BOS therapy abrogated both H/R and ET-1 induced effects. The caspase inhibitor zvad given during hypoxia attenuated caspase 9 activation following H/R with a further reduction in caspase 9 activity when zvad was administered throughout hypoxia and reperfusion. Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; BOS: Bosentan; Hyp: Hypoxia; H/R: Hypoxia and reperfusion.

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Figure 58. Caspase 9 Activity. PKC inhibition and activation resulted in greater caspase 9 activation following H/R than control. Co-incubation with ET-1 resulted in an added increase in caspase 9 activity. A reperfusion time dependent increase in caspase 9 activity was also observed. Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerthyrine; Hyp: Hypoxia; H/R: Hypoxia and reperfusion.

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Figure 59. Caspase 9 Activity. PKA modulation did not alter H/R or ET-1 induced caspase 9 activation. Akt/PKB inhibition significantly increased caspase 9 activation following H/R. Co-incubation of SH5 with ET-1 further increased caspase 9 activation. Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; FOS: Forskolin (PKA agonist); Hypoxia; H/R: Hypoxia and reperfusion.

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Figure 60. Caspase 9 Activity. BOS therapy during hypoxia and reperfusion attenuated the effects of H/R, ET and SH5 on caspase 9 activation. ET-1 treatment during both H and R enhanced caspase 9 activity after H/R compared to all other treatment groups. Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; BOS: Bosentan; BE: BOS+ET; Con/BOS: treatment with BOS during reperfusion; ET/BOS: Hypoxia with ET and treatment with BOS during reperfusion; ET/ET: ET during hypoxia and reperfusion (H/R); BOS/BOS: BOS during H/R; BE/BE: BE during H/R.

Figure 61. Autophagy. H/R did not demonstrate increased MDC fluorescence. MDC fluorescence was increased only following Akt/PKB inhibition with FOS, with no effect seen after PKC inhibition with either Cal or Chel. Legend: Con: Control; ET: Endothelin-1; BOS: Bosentan; Hypoxia and reperfusion (H/R); Cal: Calphostin C; Chel: Chelerythrine; FOS: Forskolin (PKA agonist).

Figure 62. Necrosis. H/R did not demonstrate increased PI fluorescence. No treatment group resulted in necrosis. Legend: Con: Control; ET: Endothelin-1; BOS: Bosentan; Hypoxia and reperfusion (H/R); Cal: Calphostin C; Chel: Chelerythrine; FOS: Forskolin (PKA agonist).

Figure 63. NO production in HSVEC following H/R. H/R significantly impaired NO production. ET-1 exposure during hypoxia further reduced NO release. A significant reperfusion time dependent decrease in NO production was observed in all treatment groups. Exposure to the PKC inhibitors Cal and Chel significantly lowered NO release from HSVECs after H/R. However, co-incubation of ET-1 with PKC inhibitors demonstrated no added reduction in NO levels. HSVEC exposed to PMA demonstrated increased NO production compared to control and PMA significantly abrogated ET-1 induced effects. BOS treatment was effective at blocking ET-1 effects and increased NO production during reperfusion. Legend: NO: Nitric Oxide; HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan.

Figure 64. NO production in HSVEC following H/R. PKA modulation did not alter H/R or ET-1 induced reductions in NO production. Akt/PKB inhibition significantly impaired NO production compare to Con. Co-incubation of SH5 with ET-1 further reduced NO release. Legend: NO: Nitric Oxide; HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; FOS: Forskolin (PKA agonist).
Figure 65. NO production in HSVEC following H/R. BOS treatment during reperfusion limited the effects of both H/R and ET-1. NO production demonstrated greater improvement after one hour of reperfusion compared to 24 hours. The exposure of ET-1 during reperfusion aggravated NO impairment compared to ET-1 during hypoxia alone. Legend: NO: Nitric Oxide; HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; BOS: Bosentan; BE: BOS+ET; Con/BOS: treatment with BOS during reperfusion; ET/BOS: Hypoxia with ET and treatment with BOS during reperfusion; ET/ET: ET during hypoxia and reperfusion (H/R); BOS/BOS: BOS during H/R; BE/BE: BE during H/R.

Figure 66. NO production in HSVEC following H/R. ZI exposure reduced NO production following H/R in both Con and ET-1. DI exposure during hypoxia and reperfusion limited NO impairment in both Con and ET-1 exposed groups. EI therapy during hypoxia exacerbated NO impairment following H/R with further reduction observed when EI was given during reperfusion. Legend: NO: Nitric oxide; HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; ZI: PKCλ inhibitor, DI: Rottlerin; EI: PKCε inhibitor.

Figure 67. ROS production and H/R. H/R increased ROS production compared to normoxia. ET-1 exposure during hypoxia enhanced ROS release. PKC inhibition resulted in significantly reduced ROS formation while PKC activation with PMA increased ROS production following H/R. Co-incubation of ET-1 with Cal, Chel, and PMA resulted in increased ROS production compared to Cal, Chel and PMA alone. PKA modulation did not alter H/R nor ET-1 induced ROS release. SH5 exposure increased ROS formation compared to H/R alone. BOS therapy was able to attenuate both H/R and ET-1 induced effects. Legend: ROS: Reactive oxygen species; Con: Control; ET: Endothelin-1; BOS: Bosentan; Hypoxia and reperfusion (H/R); Cal: Calphostin C; Chel: Chelerythrine; FOS: Forskolin (PKA agonist).

Figure 68. ROS production and H/R. BOS treatment during reperfusion significantly reduced ROS formation. ET-1 exposure during reperfusion increased ROS production. Legend: ROS: Reactive oxygen species; Con: Control; ET: Endothelin-1; BOS: Bosentan; BE: BOS+ET; Con/BOS: treatment with BOS during reperfusion; ET/BOS: Hypoxia with ET and treatment with BOS during reperfusion; ET/ET: ET during hypoxia and reperfusion (H/R); BOS/BOS: BOS during H/R; BE/BE: BE during H/R.

Figure 69. ROS production and H/R. Antioxidant therapy significantly reduces ROS production following H/R when given during hypoxia. Antioxidant therapy
during both hypoxia and reperfusion further lowered ROS formation. Legend: ROS: Reactive oxygen species; AO: Antioxidant; Con: Control; Sep: Sepiapterin (NOS cofactor); TI: Tiron (SOD anion); TE: Tempol (SOD mimetic).

Figure 70. ROS production and H/R. Antioxidant therapy significantly reduces ROS production following H/R when given during hypoxia in the ET-1 group. Antioxidant therapy during both hypoxia and reperfusion further lowered ROS formation after H/R in the ET-1 group. Legend: ROS: Reactive oxygen species; AO: Antioxidant; ET: Endothelin-1; Sep: Sepiapterin (NOS cofactor); TI: Tiron (SOD anion); TE: Tempol (SOD mimetic).

Figure 71. ROS production and H/R. ZI and EI exposure during hypoxia resulted in significant ROS reduction after hypoxia and reperfusion while DI lowered free radical formation only during reperfusion. When isoform specific PKC inhibition was continued into the reperfusion phase a further reduction in ROS formation was observed. In the ET-1 exposed group all isoform specific inhibitors decreased ROS production. Legend: ROS: Reactive oxygen species; No inh: No inhibitor; Con: Control; ET: Endothelin-1; ZI: PKCλ inhibitor, DI: Rottlerin; EI: PKCε inhibitor.

Figure 72. H/R and PKC activity. Hypoxia increases PKC activity. Reperfusion for both one and 24 hours increased PKC activity compared to normoxia and hypoxia. ET-1 treatment during hypoxia reduced consensus activity compared to hypoxia alone however the activity is higher than when ET-1 is given during normoxia. BOS treatment prevented the rise in PKC activity seen in the control group during H/R. Legend: Con: Control; ET: Endothelin-1; BOS: Bosentan; H/R: hypoxia and reperfusion.

Figure 73. H/R and PKA activity. No differences were seen in PKA activity following H/R. Legend: Con: Control; ET: Endothelin-1; BOS: Bosentan; H/R: Hypoxia and reperfusion.

Figure 74. Akt/PKB Western Blots. Significant differences were seen between groups Following H/R. H/R significantly reduced serine phosphorylation compared to normoxia. When ET-1 was added during hypoxia a further reduction in serine phosphorylation was seen. H/R significantly reduced threonine phosphorylation compared to normoxia. When ET-1 was added during hypoxia a further reduction in threonine phosphorylation was seen.
Figure 75. Akt/PKB Serine Phosphorylation. H/R significantly reduced serine phosphorylation compared to normoxia. When ET-1 was added during hypoxia a further reduction in serine phosphorylation was seen. BOS treatment during hypoxia limited the effect of hypoxia and one hour of reperfusion in both the control and ET-1 groups. Following 24 hours of reperfusion BOS abrogated ET-1 induced effects. PKC inhibition significantly reduced Akt/PKB serine phosphorylation following hypoxia and reperfusion. Co-incubation with ET-1 further lowered serine/total ratio during reperfusion but not after hypoxia alone. PMA decreased serine phosphorylation compared to control following 24 hours of reperfusion. An synergistic effect was seen with ET-1 co-incubation during one and 24 hours of reperfusion. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist).

Figure 76. Akt/PKB Threonine Phosphorylation. H/R significantly reduced threonine phosphorylation compared to normoxia. When ET-1 was added during hypoxia a further reduction in threonine phosphorylation was seen. BOS treatment during hypoxia limited the effects of H/R and ET-1. At 24 hours of reperfusion the BOS groups demonstrated greater threonine phosphorylation. PKC inhibition significantly reduced Akt/PKB threonine phosphorylation following hypoxia and reperfusion. PMA exposure did not alter H/R or ET-1 induced effects. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist).

Figure 77. eNOS Western Blots. Significant differences were observed between groups Following H/R. H/R significantly reduced eNOS expression following hypoxia compared to normoxia. ET-1 treatment during hypoxia further downregulated eNOS expression. BOS abrogated both hypoxia and ET-1 induced alterations in eNOS expression. During reperfusion eNOS expression increased and returned to normoxic levels after 24 hours. Akt/PKB and PKA modulation did not alter the effects of hypoxia or ET-1 on eNOS protein expression. PKC inhibition resulted in eNOS downregulation following hypoxia compare to control while PMA resulted in a slight upregulation. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist).

Figure 78. eNOS Expression. H/R significantly reduced eNOS expression following hypoxia compared to normoxia. ET-1 treatment during hypoxia further downregulated eNOS expression. BOS abrogated both hypoxia and ET-1 induced alterations in eNOS expression. During reperfusion eNOS expression increased and returned to normoxic levels after 24 hours. Akt/PKB and PKA modulation did not alter the effects of hypoxia or ET-1
on eNOS protein expression. PKC inhibition resulted in eNOS downregulation following hypoxia compare to control while PMA resulted in a slight upregulation. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist).

Figure 79. iNOS Western Blot images. No significant induction of iNOS was observed following H/R. No differences between groups were seen.

Figure 80. Caveolin-1 Western Blot images. No significant differences in caveolin-1 expression were observed.

Figure 81. Caveolin-1 Expression. No significant differences in caveolin-1 expression were observed. Legend: ROS: Reactive oxygen species; Con: Control; ET: Endothelin-1; BOS: Bosentan; Hypoxia and reperfusion (H/R); Cal: Calphostin C; Chel: Chelerythrine; FOS: Forskolin (PKA agonist).

Figure 82. PKCδ Western Blots. PKCδ translocation was significantly affected by H/R. Significant differences between groups were observed. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); H/R: Hypoxia and reperfusion.

Figure 83. PKCδ Translocation. PKCδ translocation is significantly increased following H/R. ET-1 exposure further translocated PKCδ to the membrane. PKC inhibition attenuated the effects of H/R on delta translocation. PKA and PKB modulation did not alter H/R and ET-1 induced changes in PKCδ translocation. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); H/R: Hypoxia and reperfusion.

Figure 84. PKCε Western Blots. PKCε translocation was significantly affected by H/R. Significant differences between groups were observed. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); H/R: Hypoxia and reperfusion.

Figure 85. PKCε Translocation. PKCε translocation is significantly increased following H/R. ET-1 exposure further translocated PKCε to the membrane. PKA and PKB modulation did not alter H/R and ET-1 induced changes in PKCε translocation. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C;
Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); H/R: Hypoxia and reperfusion.

Figure 86. PKCλ Western Blots. PKCλ translocation was significantly affected by H/R. Significant differences between groups were observed. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); H/R: Hypoxia and reperfusion.

Figure 87. PKCλ Translocation. PKCλ translocation is significantly reduced following H/R. ET-1 exposure further reduced the PKCλ M/C ratio. Neither PKA nor Akt/PKB modulation altered H/R and ET-1 induced changes in PKCλ translocation. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); H/R: Hypoxia and reperfusion.

Figure 88. eNOS Western Blots. Images depict eNOS cytosolic and membrane expression. Significant differences were found between groups. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); H/R: Hypoxia and reperfusion.

Figure 89. eNOS Localization. Hypoxia significantly increased the eNOS M/C ratio. ET-1 treatment during hypoxia further localized eNOS to the membrane after hypoxia. BOS therapy limited the effects of hypoxia on eNOS translocation. Akt/PKB inhibition during hypoxia demonstrated a higher M/C ratio compared to control with an additive increase in eNOS localization when co-incubated with ET-1. PKC inhibition during hypoxia increased the M/C ratio with a further increase with ET-1 co-incubation. PMA exposure revealed eNOS localization to the membrane following hypoxia and a greater membrane presence of eNOS when co-incubated with ET-1. All groups demonstrated a return to a normoxic M/C ratio following one or 24 hours of reperfusion. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist).

Figure 90. Endothelial dependent vasodilation in rat thoracic aorta. The graph depicts the cumulative dose-response curves to Ach in aortic segments. CyA treatment resulted in impaired endothelial dependent vasorelaxation compared to SRL and Con. SRL exposure improved endothelial dependent vasorelaxation compared to control. Legend: Ach: Acetylcholine; CyA: Cyclosporine; SRL: Rapamycin; Con: Control.
Figure 91. Endothelial dependent vasodilation in rat thoracic aorta. The graph depicts the cumulative dose-response curves to Ach in aortic segments. BH$_4$ treatment limited CyA induced endothelial dysfunction but failed to restore the vasculature to normal Con function. Legend: Ach: Acetylcholine; CyA: Cyclosporine; Con: Control; BH$_4$: Tetrahydrobiopterin.

Figure 92. Endothelial dependent vasodilation in rat thoracic aorta. The graph depicts the cumulative dose-response curves to Ach in aortic segments. BOS treatment completely abrogated CyA induced endothelial dysfunction. No significant differences were seen between Con and BOS treated groups. Legend: Ach: Acetylcholine; CyA: Cyclosporine; Con: Control; BOS: Bosentan.

Figure 93. Endothelial dependent vasodilation in rat thoracic aorta. The graph depicts the cumulative dose-response curves to Ach in aortic segments. Combined BOS and BH$_4$ treatment completely abrogated CyA induced endothelial dysfunction. Combined treatment increased vasodilatory response to Ach compared to Con. Legend: Ach: Acetylcholine; CyA: Cyclosporine; Con: Control; BOS: Bosentan; BH$_4$: Tetrahydrobiopterin.

Figure 94. Endothelial dependent vasodilation in rat thoracic aorta. The graph depicts the endothelial dependent vasorelaxation at maximal Ach concentration. CyA treatment resulted in the lowest Emax% compared to all experimental groups. Combined BOS and BH$_4$ groups had the highest Emax% compared to Con and all other treatment groups. Legend: Ach: Acetylcholine; CyA: Cyclosporine; Con: Control; SRL: Rapamycin; BOS: Bosentan; BH$_4$: Tetrahydrobiopterin.

Figure 95. Endothelial dependent vasodilation in rat thoracic aorta. The graph depicts the concentration of Ach required to elicit half maximal vasodilation (ED$_{50}$). CyA treatment resulted in the highest ED$_{50}$ compared to all experimental groups. BH$_4$ attenuated the CyA-induced increase in the Ach ED$_{50}$. BOS and the combined BOS and BH$_4$ groups had lower ED$_{50}$ compared to Con. SRL exposure resulted in the lowest ED$_{50}$ compared to all treated groups. Legend: Ach: Acetylcholine; CyA: Cyclosporine; Con: Control; SRL: Rapamycin; BOS: Bosentan; BH$_4$: Tetrahydrobiopterin.

Figure 96. Endothelial independent vasodilation in rat thoracic aorta. The graph depicts the cumulative dose-response curves to SNP in aortic segments. CyA treatment resulted in impaired endothelial independent vasorelaxation.
compared to SRL and Con. Legend: SNP: sodium nitroprusside; CyA: Cyclosporine; Con: Control; SRL: Rapamycin.

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Figure 97. Endothelial independent vasodilation in rat thoracic aorta. The graph depicts the cumulative dose-response curves to SNP in aortic segments. BH$_4$ treatment limits CyA induced dysfunction. Legend: SNP: sodium nitroprusside; CyA: Cyclosporine; Con: Control; BH$_4$: Tetrahydrobiopterin.

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Figure 98. Endothelial independent vasodilation in rat thoracic aorta. The graph depicts the cumulative dose-response curves to SNP in aortic segments. BOS treatment abrogates CyA induced dysfunction. Legend: SNP: sodium nitroprusside; CyA: Cyclosporine; Con: Control; BOS: Bosentan.

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Figure 99. Endothelial independent vasodilation in rat thoracic aorta. The graph depicts the cumulative dose-response curves to SNP in aortic segments. Combination therapy with or without CyA resulted in improved vasodilation to SNP than control. Legend: SNP: sodium nitroprusside; CyA: Cyclosporine; Con: Control; BOS: Bosentan; BH$_4$: Tetrahydrobiopterin.

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Figure 100. Endothelial independent vasodilation in rat thoracic aorta. The graph depicts the endothelial independent vasorelaxation at maximal SNP concentration. No significant differences were seen between Con and CyA groups. The combined BOS and BH$_4$ group as well as the BH$_4$ alone group had the highest Emax% compared to Con and all other treatment groups. Legend: SNP: sodium nitroprusside; CyA: Cyclosporine; Con: Control; BOS: Bosentan; BH$_4$: Tetrahydrobiopterin.

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Figure 101. Endothelial independent vasodilation in rat thoracic aorta. The graph depicts the concentration of SNP required to elicit half maximal vasodilation (ED$_{50}$). CyA treatment resulted in the highest ED$_{50}$ compared to all experimental groups. BH$_4$ attenuated the CyA-induced increase in the SNP ED$_{50}$. The combined BOS and BH$_4$ group had the lowest ED$_{50}$ compared to all groups. Legend: SNP: sodium nitroprusside; CyA: Cyclosporine; Con: Control; SRL: Rapamycin; BOS: Bosentan; BH$_4$: Tetrahydrobiopterin.

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Figure 102. Sensitivity to Vasospasm. Cumulative dose-response curves to ET-1 in aortic segments. CyA increased vaso sensitivity to ET-1 compared to Con and SRL. Legend: ET-1: Endothelin-1; CyA: Cyclosporine; Con: Control; SRL: Rapamycin.

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Figure 103. Sensitivity to Vasospasm. Cumulative dose-response curves to ET-1 in aortic segments. All three treatment groups (CyA, BH4+CyA, and BH4) demonstrated increased vaso sensitivity to ET-1 compared to Con. However, no significant differences were seen between CyA, BH4+CyA, and BH4 exposed rats. Legend: ET-1: Endothelin-1; CyA: Cyclosporine; Con: Control; BH4: Tetrahydrobiopterin.

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Figure 104. Sensitivity to Vasospasm. Cumulative dose-response curves to ET-1 in aortic segments. BOS treatment (with or without CyA) resulted in a reduced sensitivity to ET-1 induced vasospasm compared to Con. Legend: ET-1: Endothelin-1; CyA: Cyclosporine; Con: Control; BOS: Bosentan.

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Figure 105. Sensitivity to Vasospasm. Cumulative dose-response curves to ET-1 in aortic segments. Combined BOS and BH4 treatment (with or without CyA) resulted in a reduced sensitivity to ET-1 induced vasospasm compared to Con. Legend: ET-1: Endothelin-1; CyA: Cyclosporine; Con: Control; BH4: Tetrahydrobiopterin; BOS: Bosentan.

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Figure 106. The graph depicts vasoreactivity at maximal ET-1 concentrations. CyA treatment resulted in a greater %Cmax compared to Con. The combined BOS and BH4 group had the lowest %Cmax compared to Con and all other treatment groups. BH4 exposure (with or without CyA) resulted in increased vasospasm. Legend: ET-1: Endothelin-1; CyA: Cyclosporine; Con: Control; BH4: Tetrahydrobiopterin; BOS: Bosentan.

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Figure 107. The graph depicts the concentration of ET-1 required to elicit half maximal vasocontraction (ED50). CyA treatment resulted in a reduced ED50 compared to Con. BH4 exposure also lowered the ED50 compared to Con. BOS and the combined BOS and BH4 groups increased the ED50 compared to all experimental groups. Legend: ET-1: Endothelin-1; CyA: Cyclosporine; BH4: Tetrahydrobiopterin; BOS: Bosentan.

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Figure 108. Plasma ET-1 Levels. CyA exposure did not alter plasma ET-1 levels compared to control. SRL treated animals demonstrated a significantly lower plasma ET-1 level. BH4 and BH4+CyA treatment resulted in reduced ET-1 plasma levels compared to both control and CyA treated animals. BOS exposure also reduced plasma ET-1 levels. Legend: ET-1: Endothelin-1; CyA: Cyclosporine; Con: Control; BH4: Tetrahydrobiopterin; BOS: Bosentan.

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Figure 109. This figure displays 8-isoprostane levels as a percent change from baseline. CyA increased ROS injury compared to all groups. BOS therapy significantly abrogated CyA-induced oxidative injury. BH4 completely attenuated CyA free radical injury while BH4 alone reduced ROS injury to below control levels. Legend: ROS: Reactive oxygen species; Con: Control; CyA: Cyclosporine; SRL: Rapamycin; BOS: Bosentan; BH4: Tetrahydrobiopterin; BB: BOS+BH4.

Figure 110. This figure demonstrates ROS production as a percentage of control in human saphenous vein endothelial cells. A. CyA increased ROS production compared to all groups. BOS therapy significantly abrogated CyA oxidative burst. BOS exposure lowered free radical production compared to Con. B. BH4 partially attenuated CyA induced ROS production while BH4 alone reduced free radical release to below control levels. Legend: ET-1: Endothelin-1; CyA: Cyclosporine; Con: Control; BH4: Tetrahydrobiopterin; BOS: Bosentan.

Figure 111. A. Western Blot for ETₐRc expression demonstrated upregulation following CyA treatment. B. Quantitative Western blot analysis of ETₐRc expression in the thoracic aorta. CyA treatment increased ETₐRc expression compared to SRL and Con. BH4 and BOS therapy abrogated CyA-induced upregulation. Legend: Rc: Receptor; Con: Control, Cyclosporine: CyA; SRL: Rapamycin, BH4: Tetrahydrobiopterin, BOS: Bosentan, BB: BOS+BH4.

Figure 112. A. Western Blot for ETₐ receptor Rc expression demonstrated upregulation following BH4 treatment. B. Quantitative Western blot analysis of ETₐRc expression in the thoracic aorta. BH4 treatment increased ETₐRc expression compared to all treatment groups. BOS therapy abrogated BH₄-induced upregulation. Legend: Rc: Receptor; Con: Control, Cyclosporine: CyA; SRL: Rapamycin, BH₄: Tetrahydrobiopterin, BOS: Bosentan, BB: BOS+BH₄.
Figure 113. A. i-iv) Western Blot for eNOS protein expression demonstrated downregulation following CyA treatment. BH4 and BOS exposure limits CyA-induced downregulation while combined therapy restores normal eNOS expression. v) Western Blot for iNOS protein expression demonstrating no differences between groups. B. Quantitative Western blot analysis of eNOS expression in the thoracic aorta. Legend: Con: Control, Cyclosporine: CyA; SRL: Rapamycin, BH4: Tetrahydrobiopterin, BOS: Bosentan, BB: BOS+BH4.

Figure 114. A. Western Blot for TNF-α protein expression demonstrated no significant differences between groups. B. Quantitative Western blot analysis of TNF-α expression in the left ventricle revealed no differences between groups. Legend: Con: Control, Cyclosporine: CyA; SRL: Rapamycin, BH4: Tetrahydrobiopterin, BOS: Bosentan, BB: BOS+BH4, TNF-α: Tumor necrosis factor-α.

Figure 115. A. Western Blot for TGF-β protein expression demonstrated no significant differences between groups. B. Quantitative Western blot analysis of TGF-β expression in the left ventricle revealed no differences between groups. Legend: Con: Control, Cyclosporine: CyA; SRL: Rapamycin, BH4: Tetrahydrobiopterin, BOS: Bosentan, BB: BOS+BH4, TGF-β: Transforming growth factor-β.

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LEGENDS FOR TABLES

Table 1: Comparison between CAV and CAD.

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Table 2: Summary of vascular effects. CyA: Cyclosporine; SRL: Rapamycin; BH₄: Tetrahydrobiopterin; ET-1: Endothelin-1; Ach: Acetylcholine; SNP: sodium nitroprusside; ROS: Reactive Oxygen Species; eNOS: endothelial nitric oxide synthase; ET-1Rc: ET-1 receptor (either A or B) Emax%: % maximal relaxation, %Cmax: % maximal contraction.

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INTRODUCTION

Heart failure remains the only cardiovascular diagnosis increasing in incidence [1;2]. The accepted therapy for end stage heart failure is cardiac transplantation; however, the increasing number of eligible patients far outstrips the number of available organs. Those patients fortunate enough to receive a heart transplant still suffer from early and late morbidity. Early morbidity is predominantly the result of primary graft dysfunction attributable to ischemic injury sustained at the time of organ procurement. Late phase morbidity results from 3 main causes; infection, lymphoproliferative disease, and cardiac allograft vasculopathy (CAV). Angiographic studies have documented CAV in up to 45% of transplant recipients as early as 3 years following transplantation [3;4].

Known risk factors for coronary artery disease, such as hypertension, hypercholesterolemia, hyperhomocysteinemia (HHcy) and hyperglycemia influence the progression of CAV; however, several other unique features of cardiac transplantation have a greater impact on the development of CAV. For example, recent evidence has suggested that ischemic injury to the endothelium sustained at the time of organ procurement and storage may be related to the development and progression of CAV [5-7]. Therefore, improved methods of allograft protection specifically aimed towards endothelial preservation have the potential to reduce the incidence of both early graft dysfunction and late CAV. Verrier et al. demonstrated in a rodent model of orthotopic aortic allograft transplantation that ischemia and reperfusion (I/R) results in endothelial injury leading to the development of transplant vasculopathy [8]. They also found endothelial cell loss occurred in both isografts and allografts due to I/R [8]. This initial
loss of endothelial cells was replaced by two weeks; however transplant vasculopathy developed within 60 days [8]. Furthermore, their study indicated that I/R injury led to the development of transplant vasculopathy since isografts developed vasculopathy, although to a lesser extent then the allografts. Poston et al. demonstrated in their rodent heterotopic heart transplant model of vascular disease that induction of a donor inflammatory state before harvest worsened reperfusion injury and chronic graft vascular disease after transplantation [9]. Furthermore, we can infer from their study that ischemic injury promoted chronic vascular disease. Therefore, improved methods of endothelial protection/preservation have the potential to reduce the incidence of both primary graft dysfunction and CAV.

Following hypoxia, several changes occur in the endothelial cell such as a decrease in nitric oxide (NO) production and an increase in endothelin-1 (ET-1) release [10-12]. NO is produced by transforming one molecule of L-arginine to L-citruline and NO. The enzyme responsible for this reaction is nitric oxide synthase (NOS). ET-1 is produced by endothelial cells. Endothelial cells release ET-1 as preET-1 and an endothelin converting enzyme (ECE) present on pulmonary capillaries converts preET-1 to ET-1. ET-1 is one of the most potent endogenous vasoconstrictors and mediates a host of responses including endothelial dysfunction, vasomotor contraction, leukocyte activation and cellular proliferation. Additionally, it augments the vascular actions of other vasoactive substances such as angiotensin-II and norepinephrine. ET-1 exerts its biological effects via binding and activating ET-1 receptors (ET\textsubscript{A} and ET\textsubscript{B}). Diminished production of NO and exaggerated release of ET-1 are believed to be key initiators of
endothelial injury. ET-1 also results in leukocyte activation and production of reactive oxygen species (ROS).

In-vitro studies have demonstrated that I/R activates the endothelial cell leading to the expression of cell surface adhesion molecules such as intra-cellular adhesion molecules (ICAM), vascular cell adhesion molecule (VCAM) and E-selectin, among others [13;14]. In addition, activated endothelial cells produce and release cytokines such as interleukin-1 (IL)-1 and IL-8, which promote the adhesion of circulating leukocytes in conjunction with the increased expression of cellular adhesion molecules. I/R activates leukocytes leading to the expression of CD11 and L-selectin on neutrophils, activating macrophages, and increasing cytokine production (IL2 and IL6). During reperfusion, circulating leukocytes bind to the activated endothelial cells, migrate through the endothelial layer into the media and start to produce oxygen derived free radicals and toxic cytokines such as tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β). The production of superoxide radicals significantly increases endothelial injury. Therefore, if I/R injury can be prevented, it may be possible to obviate much of the myocardial and endothelial dysfunction seen early following cardiac transplantation. Early endothelial injury and activation may also predispose the vasculature to persistent immune mediated injury. Since one proposed mechanism for the development of CAV is chronic vascular rejection, prevention of early endothelial injury may delay the progression of CAV.

The need for postoperative immunosuppression is also linked to the development of CAV and may be due to direct damage to the endothelium with resulting vascular dysfunction [3]. A novel therapeutic strategy that can be applied at the time of
transplantation and continued into the posttransplant period would therefore provide a
tremendous benefit to heart transplant recipients. Since CAV is pathophysiologically
similar to disease processes in other solid organ transplant recipients, such as
bronchiolitis obliterans in lung grafts, foam cell arteriopathy in liver grafts and chronic
allograft nephropathy in renal grafts, a novel strategy to preserve vascular function would
have important implications for a wide variety of patients [6;7].

Bosentan (BOS) is a non-selective ET-1 antagonist which we and others have shown
to confer cellular protection against I/R [15-17]. We have previously shown in a human
cardiomyocyte cell model of simulated I/R that ET-1 antagonism improves cell survival
[18]. Furthermore, elevated ET-1 levels worsen I/R injury and results in impaired
vascular function as assessed by videomicroscopy of atrial arterioles [16]. In addition,
ET-1 antagonism attenuated ischemic injury and improved vasomotor function [16].
Extending these observations in a large animal model of orthotopic transplantation, we
were able to demonstrate that ET-1 blockade significantly improved recovery of both
endothelial and myocardial function following 6 hours of allograft storage [17]. In this
study, endothelial function (% maximal vasodilation (Emax%) to acetylcholine (Ach)) of
coronary vessels was improved by treatment with BOS. BOS treated animals had an
Emax% of 71± 4% after cardioplegic arrest compared to an Emax% of 65±7% for control
animals (p=0.1). When examining coronary vessels after orthotopic transplantation we
found that I/R resulted in endothelial dysfunction in both groups (control Emax%: 33±
3% and BOS Emax%: 55± 5%) but that BOS supplementation limited endothelial injury
compared to control (p<0.05). When coronary vessels were exposed to sodium
nitroprusside (SNP) no significant differences were seen in vasomotor function. These
results indicated that BOS treated animals had improved vascular function as a result of improved preservation of endothelial function. Furthermore, BOS resulted in decreased free radical injury following I/R.

Gonon and colleagues demonstrated that NO mediates the protective effect of BOS in isolated rat hearts [19]. NO has been extensively investigated by our laboratory and others and has proven to be an important component to vascular stability following I/R [12;20-31]. The added insult of transplant immunosuppression may further complicate the interaction between ET-1 and NO and lead to exacerbation of transplant vasculopathy. Furthermore, recent evidence links the antiproliferative effects of rapamycin (SRL) to increased NO bioavailability [20]. However, little data exists investigating the relationship between hypoxia, ET-1, NO and SRL and the mechanism of vascular injury in transplant recipients.

We aimed to elucidate the mechanism of endothelial injury, specifically as it applies to the post-transplant setting which is associated with elevated ET-1 levels, and direct cytotoxic injury secondary to immunosuppressive therapy [32;33]. We employed an isolated cell culture model to assess the effects of various interventions on human endothelial cells. Our studies provide fundamental data to support the use of targeted pharmacological therapy for the prevention of endothelial dysfunction and will hopefully improve both the short and long term results of cardiac transplantation.
CHAPTER ONE

KNOWLEDGE TO DATE
1.1 CARDIAC ALLOGRAFT VASCULOPATHY

Heart transplantation is the accepted standard therapy for patients with refractory end-stage heart disease. While this surgical procedure is able to extend and improve quality of life, it is not a cure. The median survival following heart transplantation remains 9.3 years and 11.8 years for patients surviving the first year post-transplantation [34]. CAV is the leading cause of mortality between 1 and 3 years post-transplant according to The Registry of the International Society for Heart and Lung Transplantation [34]. After year 3, CAV accounts for 17% of deaths. Angiographic studies indicate that CAV occurs in 42% of all heart transplant patients 3 years following transplantation [34]. Intravascular ultrasound, a more sensitive technique, detects CAV in 75% of patients at 3 years. Allograft vasculopathy is a phenomenon not limited to cardiac transplantation. A similar process also limits long term graft survival in other solid organ transplants.

CAV is an accelerated form of coronary artery disease (CAD) that affects arteries, arterioles, capillaries, and occasionally veins, with sparing of all recipient vessels [35-37]. The predominant feature of CAV is a diffuse and progressive thickening of the arterial intima that develops in both the epicardial and intramyocardial arteries of the transplanted heart. The process is a concentric fibrous intimal hyperplasia that appears along the entire length of the affected arteries. Included in this form of arteriosclerosis are features of both atherosclerosis and arteritis. The atherosclerotic changes range from a diffuse incorporation of lipids to the development of classic focal plaques later in the disease process. With arteritis, there is thickening of the vessel due to infiltration by mononuclear inflammatory cells responding to alloimmune or infectious stimuli. Rarely, this arteritis may progress to cause destruction of the internal elastic lamina and involvement of the media [38-41].
1.1.1 Differences between CAV and CAD

The pathologic features of CAV differ significantly from those of CAD (Table 1). CAD is usually a focal and eccentric proliferation of the intima of proximal coronary vessels. There is usually sparing of the intramyocardial vessels. Fatty streaks are the initial lesions seen. Of importance in CAD is the deposition of calcium ($\text{Ca}^{2+}$) and disruption of the elastic lamina. Rarely there are signs of inflammation, and veins are never involved.

CAV is typically characterized as a diffuse concentric proliferation of the intima. Intramyocardial vessels are usually involved and the process can even involve the coronary veins. The initial lesions seen are smooth muscle proliferation of the intima. Of note, there is rarely any $\text{Ca}^{2+}$ deposition [38], the internal elastic lamina (IEL) is intact, and inflammation is usually present.

1.1.2 Pathology

CAV is mainly a disease of the intima. Changes in the intima can be seen as early as 6 months post-transplant. The lesion at this time is a mild intimal thickening. Mild fibrosis and increases in extracellular matrix proteins may be present. Early post-transplant, intimal thickening is limited to the proximal arteries [42]. These lesions are characterized by hyperplastic fibrous thickening. Lesions then progress to a fibrofatty atheromatous plaque. Ultimately, the coronary vasculature progresses to a diffuse fibrous thickening of the intima, which can have superimposing atheromatous plaques.

The IEL is almost always intact except for breaks, which may be seen in more advanced stages of CAV. The involvement of the media can vary from being unaffected to nearly completely replaced by fibrous tissue. As the severity of the intimal disease
progresses, so does fibrosis of the media. The only vessels relatively unaffected are those with little or no muscular layer.

1.1.3 Pathophysiology

The pathophysiology of CAV, although not completely understood, likely involves components of both immune-mediated and non-immune-mediated endothelial damage, and passenger “native vessel” atherosclerosis [43]. There is substantial evidence that immunological factors, including histocompatibility mismatch, acute rejection episodes, and chronic inflammation play a major role in the development of CAV. Nonimmunological factors include cause of donor brain death, cytomegalovirus (CMV) infection, age, sex, obesity, dyslipidemia, HHcy, diabetes, hypertension, smoking, and I/R injury [18]. In general, hyperlipidemia and insulin resistance are the most significant nonimmunological factors, occurring in 50-80% of the heart transplant population [44].

The endothelial damage involved in CAV can be categorized into either denuding or nondenuding injury. In nondenuding injury a rapid replacement of injured endothelial cells leads to endothelial dysfunction [41]. Both immune-related and non-immune-related factors contribute to nondenuding injury. In contrast, denuding injury is caused by I/R injury during the transplant process or during episodes of acute cellular rejection. This results in the loss of large stretches of endothelium along the vessel, which causes significant endothelial dysfunction. According to one hypothesis, it is the immune component or alloantigen-dependent mechanism of injury that acts principally to intensify initial non-immune damage to the endothelial cells [45;46]. Denuding injury allows for blood components and circulating cytokines to have direct contact with the subintimal layers. This can lead to significant proliferation of smooth muscle cells (SMC). Therefore, CAV can be
initiated or exacerbated by several processes that can lead to denuding or non-denuding injury. These include I/R injury, immune activation, viral infection, and injury from immunosuppressive drugs.

Hyperlipidemia is commonly seen in cardiac transplant patients for several reasons. Many cardiac transplant patients are hyperlipidemic prior to transplantation. In addition, the immunosuppressive therapy given to patients, especially calcineurin inhibitors, may result in or exacerbate pre-existing dyslipidemia. Hypercholesterolemia, in a rabbit heterotopic cardiac transplant model, has been shown to be associated with CAV [47;48] and transplanted coronary arteries were more affected by hypercholesterolemia than native coronary arteries. Hypercholesterolemia promotes fibrofatty proliferative changes to the intimal hyperplasia seen in most patients with CAV [47].

In solid organ transplant recipients, HHcy is extremely common and occurs early with an incidence as high as 80-90% [49-60]. HHcy can damage cells by several mechanisms, but primarily by affecting the endothelium [61-63]. HHcy results in reduced endothelial NO production [64;65], impaired arterial response to vasodilators and increased expression of procoagulant factors [56;61-63]. The neutrophil-endothelium interaction is promoted in the setting of HHcy, allowing for the presence of more neutrophils in the intima. All of these alterations in the endothelial wall are caused by alterations in the redox state induced by high homocysteinemia (Hcy) levels [61;62;64]. Several investigators have demonstrated that HHcy is associated with the development of CAV [50;66].

Hypertension, smoking, diabetes and other risk factors for atherosclerosis are associated with CAV. Hypertension in transplant patients can be present preoperatively or
postoperatively secondary to immunosuppressive medication, such as cyclosporine (CyA). Hypertension causes endothelial injury by promoting the formation of intimal hyperplasia, which eventually leads to atherosclerotic lesions.

While there remains debate over the relative importance of the direct versus the indirect pathway of alloreactivity, one theory is that direct activation of recipient CD4\(^+\) T-cells by donor allograft/nonself major histocompatibility complex (MHC) class II molecules initiates graft rejection. CD8\(^+\) T-cells can become activated by previously activated CD4\(^+\) T-cells through the CD40L pathway and by nonself MHC class I molecules.

The activation of CD4\(^+\) and CD8\(^+\) T-cells, leads to further synthesis of cytokines, which perpetuate the ongoing cascade of events that lead to CAV. The most important cytokines in allograft rejection are IL-2, interferon-\(\gamma\) (IFN-\(\gamma\)), and TNF-\(\alpha\). IL-2 induces T-cell proliferation and differentiation, IFN-\(\gamma\) activates macrophages, and TNF-\(\alpha\) itself is cytotoxic to the transplanted heart. In addition, TNF-\(\alpha\) acts to increase MHC class I expression, while IFN-\(\gamma\) increases the expression of both MHC class I and II molecules. Overall, these cytokines can lead to chronic graft rejection. IFN-\(\gamma\) and TNF-\(\alpha\) also induce the leukocyte adhesion molecule VCAM-1, which promotes monocyte adhesion and entry through the endothelium leading to CAV.

Ardehali et al used a murine CAV model with a compromised indirect alloreactivity pathway to show that this did not affect the extent of intimal thickening or lymphocyte and macrophage infiltration following heart transplantation as compared to wild type mice [67]. Two potential explanations have been proposed by this group: (1) an impaired indirect pathway is enough to cause severe CAV and that the direct pathway
does not play a major role in CAV; or (2) the direct pathway of alloreactivity can fully compensate for the impaired indirect pathway [67]. Other studies such as those by Game et al. have found a correlation to exist between increased indirectly activated T-cells and chronic rejection [68]. As far as the importance of CD4$^+$ versus CD8$^+$ lymphocytes, it appears that the CD4$^+$ allore cognition pathway is required for CAV development, while the CD8$^+$ pathway may act to increase the severity of CAV [69;70]. In a study by Szeto et al., hearts transplanted into CD8$^+$ T cell-depleted rats developed CAV, while there was no CAV in the CD4$^+$ T cell-depleted recipient [71]. These findings suggest that CAV is dependent on CD4$^+$ indirect allore cognition and not a CD8$^+$ direct pathway. It remains controversial as to which component of the immune response is involved in CAV but most experts agree that immune activation plays a role in the development of CAV.

Acute rejection as a cause or risk factor for CAV has been investigated by several authors [72-75]. Some groups have reported an association between the severity and frequency of rejection and the severity of CAV, however others have reported that episodes of acute rejection are not associated with the development of CAV [72-76]. One proposed mechanism linking acute rejection to CAV is that the inflammatory process and tissue destruction from rejection results in endothelial damage, which initiates the process of CAV and/or potentiates the CAV already in progress.

Recent research has correlated I/R injury with CAV. Determinants of I/R injury are length of ischemic time and methods of allograft storage. Verrier et al. demonstrated in a rodent model of orthotopic aortic allograft transplantation that I/R results in endothelial injury leading to the development of transplant vasculopathy [8]. They also found endothelial cell loss occurred in both isografts and allografts due to I/R [8]. This initial loss
of endothelial cells was replaced by two weeks; however transplant vasculopathy developed within 60 days [8]. Their study indicated that I/R injury led to the development of transplant vasculopathy since isografts developed vasculopathy, although to a lesser extent than the allografts.

Several changes occur to the endothelium following hypoxia, including loss of the ability to release NO within minutes following reperfusion [8;21;25;77]. This loss is related to the consumption of NO by superoxide radicals formed early during reperfusion [24]. Experimental evidence suggests that the oxygen free radicals are produced by neutrophils [24;78]. In vitro exposure of coronary arteries to oxygen radicals produces endothelial dysfunction [24;78]. In addition, I/R causes the endothelial cells to become activated and express surface adhesion molecules. These surface adhesion molecules promote circulating leukocyte adhesion which then cause endothelial damage by direct cytotoxicity [12;79;80]. These leukocytes become activated and release cytokines, which enhance leukocyte and SMC proliferation and activation. Ischemia also promotes complement activation that causes not only cell lysis, but results in several other changes such as increased vessel permeability, leukocyte chemoattraction, and smooth muscle contraction [81;82]. C1q can increase platelet procoagulant activity which can enhance CAV by the formation of thrombus, but mostly by causing the release of several vasoactive substances and growth factors such as platelet derived growth factor (PDGF), TGF-β, thromboxane A and prostacyclin [81]. These are few of the several mechanisms by which I/R initiates the process of CAV. In a recent study, myocardial ischemia complicated by fibrosis in the peritransplant period was associated with increased progression of CAV and a poorer long-term outcome [83].
Several investigators have reported an association between pathogens (Chlamydia pneumonia, CMV, herpes simplex and parvovirus) and CAV. Subramanian et al have demonstrated that Chlamydia pneumonia infection is correlated with the severity of CAV [84]. They concluded that CAV developed in heart transplant recipients that tested positive for IgG against Chlamydia pneumonia but not in those that tested positive for Chlamydia pneumonia by polymerase chain reaction [84]. Again, this finding implicates an immunologic mechanism behind the development of CAV, regardless of the inciting stimulus. CMV infection has been associated with both atherosclerosis and CAV. The Stanford group demonstrated that approximately 30% of CMV infected heart transplant recipients developed severe CAV representing a three fold increase compared to uninfected recipients [85]. CMV has the ability to infect vascular endothelial cells and induce endothelial injury which can lead to CAV. Weis et al reported elevated asymmetric dimethylarginine (NOS inhibitor) in CMV infected patients, thus impairing vascular homeostasis [86]. These higher levels can lead to endothelial dysfunction and correlate with increased severity of CAV [86]. CMV and herpes simplex viruses induce the host adaptive immune response, which leads to the release of cytokines, increased expression of adhesion molecules and activation of T-cell responses. Therefore, viral infection may result in CAV by impairing NO homeostasis, induction of pro-inflammatory cytokines, and enhancement of T-cell mediated alloreactivity.

The incidence of significant donor CAD remains low at approximately 2%. Donor CAD can serve as a starting point for CAV and may accelerate the disease process. Donor CAD can be important in the prognosis of the transplant patient in that it can progress independently of the CAV process. However, Botas et al, found there was no significant
difference in the rate of intimal thickening between patients with donor hearts having pre-existent coronary artery disease and those without [87]. Thus the impact of native vessel atherosclerosis on CAV remains controversial.

Finally, cause of donor brain death, more specifically explosive donor brain death (versus gradual brain death), causes an up regulation of MHC class I and II antigens, adhesion molecules and cytokine secretion, setting off an accelerated inflammatory response in the heart [88-90]. Therefore, CAV is a complex disease with a multifactorial etiology and several methods must be utilized to prevent the initiation and progression of CAV (Figure 1).

1.1.4 Diagnosis

Cardiac denervation at the time of heart transplantation usually prevents transplant patients from experiencing angina, which is an important warning sign for heart disease. Only ten to thirty percent of heart transplant patients regain any innervation to the heart. Due to this lack of early clinical symptoms, heart transplant patients with CAV typically present late with silent myocardial infarction, loss of allograft function, or sudden death [38].

Another difficulty faced by clinicians in diagnosing CAV is coronary remodeling and the diffuse nature of the disease. Angiography measures luminal diameter and compares the narrowing at plaques to normal reference diameters and previous angiograms in order to understand the severity and rate of disease progression. CAV, however, shows no initial decrease in luminal diameter due to vascular remodeling [88]. Only when the process is more advanced does the lumen narrow and angiographic detection becomes possible. Since CAV involves the entire coronary arterial tree, angiography may convey the impression of less-than-actual vessel narrowing at plaque sites. Thus angiography, while a good screening
tool for CAD, often underestimates CAV and in some patients with evenly distributed
disease throughout the coronary tree, CAV can be missed altogether [91].

Despite the poor sensitivity of angiography, it is still widely used as a screening
test for vascular disease. Johnson et al developed a classification system based on the
varying morphologies in CAV to aid in its diagnosis using angiography [42]. Briefly, Type
A lesions appear as discrete proximal tubular stenoses, Type B as diffuse concentric middle
or distal stenoses with Type B₁ as having an abrupt narrowing and Type B₂ as having a
smooth concentric tapering. Finally a Type C angiographic appearance indicates irregular
vessels with distal lesions and loss of small branches. Diagnosis of CAV requires Type B or
C lesions and comparison with previous and recent angiograms to note disease progression
[43].

A more sensitive tool is intravascular ultrasound (IVUS). IVUS is useful for
detecting the extent of intimal thickening by imaging vessel wall structure rather than simply
luminal diameter. IVUS has an axial resolution of 50-80 µm [88], unfortunately, it is
physically restricted to the larger epicardial arteries and thus cannot be used to screen for
CAV throughout the entire heart. One year post-transplant, IVUS detects CAV in 50% of
patients while angiography only detects disease in 10-20% of patients [92;93].

With IVUS, normal coronary intimal thickness ranges between 0.10 and 0.30 mm.
Hence CAV is considered present when intimal thickness exceeds 0.3mm or when the sum
of the intimal and medial thickness exceeds 0.5mm. At greater than 0.6 mm intimal
thickening, patients are 10 times more likely to experience a cardiac event [94].

Since angiography and IVUS are invasive tests, they impose increased risks upon
the patient [95]. Dobutamine stress echocardiography (DSE) is currently the most sensitive
noninvasive test for cardiac disease. DSE measures wall motion and can detect CAV with a sensitivity and specificity of 79% and 83%, respectfully [96]. Possible future modalities include both pulse-wave tissue Doppler imaging and electron-beam computed tomography. Since both modalities are non-invasive they may replace angiography as screening tools allowing IVUS to be used in high-risk patients or those with equivocal or positive test results.

1.1.5 Treatment and Prevention

Treatment of established CAV in humans remains limited. Encouraging research, however, has been done in small animals. For example, treatment with anti-CD154 in a rat cardiac allograft rejection model prevents acute rejection and drastically slows the development of CAV. In this study early treatment was required to inhibit CAV [97].

In clinical heart transplantation the focus remains on prevention of CAV via attenuation of adverse nonimmunological and immunological reactions. Prior to transplant, preventing endothelial injury at brain death, reducing cold ischemic time and subsequent tissue damage, and improving myocardial preservation during storage and transportation of the graft all aid in post-transplant cardiac function and longevity. In a study on prolonged cold storage, Kevelaitis et al demonstrated that longer cold ischemic times produced greater endothelial dysfunction in cardiac allografts [98] and that the composition of the storage medium affected the extent of allograft tissue damage. Our group has shown that profound hypothermic storage results in depressed myocardial metabolic and functional recovery [99] and that shed donor blood perfusion can permit cardiac allograft storage at tepid temperatures resulting in improved myocardial performance [99;100]. We have also shown that the addition of insulin to the blood perfusate during storage results in improved
functional and metabolic recovery during heart transplantation [101]. Fedak et al. demonstrated that BOS added to donor shed blood perfusion improves the functional recovery of both the myocardium and endothelium [17]. Several others groups have demonstrated that ET-1 antagonism reduces CAV [102;103].

Immediately following transplant, patients are placed on calcineurin immunosuppressive drugs (CyA or Tacrolimus). Unfortunately, CyA in high doses and for long periods of time can cause side effects such as renal failure [35] and hypertension. Simonson et al demonstrated in a Lewis to Fischer rat heart transplant model, that the combination of low-dose CyA with an ECE inhibitor resulted in long-term survival of the graft equal to that of high-dose CyA [104] alone. As an alternative to using CyA, other immunosuppressive drugs such as Mycophenolate Mofetil, SRL or Leflunomide, may inhibit CAV by limiting SMC proliferation [35]. The newer immunosuppressive agent Everolimus has recently been demonstrated to reduce the frequency and severity of CAV [105] in humans. Kobashigawa et al. demonstrated in patients on CyA and corticosteroids, Everolimus reduces intimal thickness and index compared to Azathioprine [105].

Hyperlipidemia is known to be a risk factor for both CAD and CAV. Unfortunately, immunosuppressive therapy with corticosteroids, CyA, SRL, and to a lesser extent Tacrolimus and Everolimus results in hyperlipidemia [106]. To treat hyperlipidemia in post-transplant patients, lipid-lowering drugs are prescribed since lifestyle changes are usually not enough to lower lipid profiles to desired levels. HMG-CoA reductase inhibitors, or statins, are the most popular and are very effective at lowering total cholesterol, low-density lipoprotein (LDL), very low density lipoprotein (VLDL), and increasing high density lipoprotein (HDL). Recently it has been documented that statins have pleiotropic
effects in that they improve vascular function. Statins decrease endothelial dysfunction via increasing NO production, inhibiting the coagulation cascade and limiting oxidized-LDL mediated damage to the endothelium [107-109]. Verma et al have also demonstrated that statins can limit cardiomyocyte injury [110]. They showed in isolated human cardiomyocytes (HCM) submitted to hypoxia and reperfusion that pravastatin reduces injury [110]. NO and ET-1 homeostasis was preserved in HCM exposed to pravastatin [110]. Pravastatin is the most commonly used HMG-CoA reductase inhibitor post heart-transplant. In 1995, Kobashigawa et al showed that treatment with Pravastatin (40mg per day) for one year, lowered mean LDL and triglyceride levels, raised HDL levels, and reduced intimal thickening and cardiac rejection accompanied by hemodynamic compromise [111] (Figure 2). In this trial, patients treated with Pravastatin had a lower incidence of coronary vascular disease and improved survival [111]. These effects may be enhanced through immunosuppression modulation since a subgroup of patients on Pravastatin had significantly reduced cytotoxicity of natural killer cells [111]. Simvastatin, likewise, has beneficial lipid-lowering effects in heart transplant recipients [112]. In addition, Simvastatin inhibits proliferation and induces apoptosis of vascular SMCs [113]. However, Simvastatin has a low but significant rate of rhabdomyolysis and myositis and thus Keogh et al proposed that Pravastatin be the statin of choice in heart transplantation [112]. Atorvastatin has been shown to further reduce LDL in heart transplant recipients with Pravastatin or Simvastatin resistance. However, of the 48 patients who had received a mean Atorvastatin dose of 21±10 mg, two developed myositis and myalgias appeared in another two patients. The study concluded that the drug was safe in moderate doses with careful patient monitoring [114].
HHcy in the cardiac transplant patient affects long-term outcomes by leading to the development of CAV. Several investigators have demonstrated that folic acid and vitamin B12 supplementation can significantly reduce Hcy levels in the cardiac transplant patient [115-117]. Kutschka et al demonstrated that folic acid supplementation (5 mg per day) can provide an effective measure to lower elevated Hcy levels in heart transplant recipients [115]. Unfortunately, these studies reveal only that Hcy levels can be lowered but do not demonstrate if reduction leads to decreased severity or prevalence of CAV.

There is general acceptance that alloimmunity plays a role in CAV. The occurrence of CAV increases as the number of HLA mismatches increases [118;119]. Prior to transplant most patients have a panel reactive antibody test performed (PRA). A PRA result of greater than 10% is considered positive and indicates that the recipient will be at higher risk of developing graft rejection. Kerman et al demonstrated that recipients with PRAs greater than 10% had a two-fold increase risk of developing CAV [120]. Immune modulation to lower PRAs has the potential to reduce acute rejection and may limit the development of CAV. Treatment strategies to lower PRAs include intravenous immunoglobulin, plasmapheresis, cyclophosphamide, mycophenolate mofetil and azathioprine. The optimal strategy to prevent alloimmune injury would be to induce tolerance. Host tolerance to the allograft will abolish rejection and the immune component of CAV development. Although, not achieved clinically, several investigators have demonstrated that tolerance can be induced in experimental models [121-124].

Once CAV has been established, treatments such as coronary angioplasty, coronary stenting, and coronary bypass surgery offer only palliative solutions. The only true solution to severe CAV is cardiac re-transplantation. Despite re-transplantation, CAV is
likely to recur and there are significant moral and ethical issues that complicate re-transplantation [38;125].

1.1.6 Summary

CAV is the major limiting factor for long-term survival following cardiac transplantation. It affects up to 75% of patients 3 years after transplantation. The risk factors for CAV can be divided in two categories (immunological and nonimmunological). Immunologic factors include the severity and frequency of acute rejection and chronic rejection. Nonimmune factors include the classic risk factors for CAD, I/R injury during organ retrieval and transplantation, CMV infection and endothelial injury from immunosuppressive drug therapy. Current areas of research focus on determining the etiology of CAV and the development of treatment strategies to prevent or limit the extent of CAV. Strategies to prevent or limit the development of CAV include: endothelial protection during organ retrieval, limiting the use of calcineurin inhibitors, and aggressive management of CAD risk factors. Other strategies include the restoration of NO and ET-1 homeostasis by utilizing NO donors, NOS cofactors or antagonizing ET-1. Another challenge in the management of CAV is its diagnosis. Early diagnosis of CAV will lead to earlier treatment and better outcomes. Angiography - the gold standard for the diagnosis of CAD - lacks the sensitivity in detecting CAV, while IVUS (the most sensitive method) lacks the ability to assess the entire coronary tree. New diagnostic tools are required for the more accurate and earlier diagnosis of CAV. The successful long-term survival of the cardiac transplant patient rests in our ability to understand, detect, treat and prevent CAV.

In conclusion, CAV is a multifactorial disease that remains the major limitation to long-term survival post-transplantation. Methods of diagnosis have improved
significantly with the use of IVUS in addition to angiography. Since treatment of CAV is limited and usually involves re-transplantation, prevention of immunological and nonimmunological risk factors is of critical importance. CAV is conceptually very similar to post-transplant pathologies in other organs (Bronchiolitis obliterans with organizing pneumonia (BOOP), biliary cirrhosis, etc). Therefore, novel therapeutic strategies to prevent or attenuate the development of CAV may have clinical relevance to several other solid organ transplant recipients.

1.2 ISCHEMIA AND REPERFUSION

Ischemia is the reduction of oxygen and nutrient supply to a tissue or organ. Reperfusion is the return of nutrient flow to the ischemic tissue or organ. Ischemia results in well-known injury to the heart with resultant reduction in cardiac function. It is imperative that reperfusion to the heart occurs as soon as possible. However, several deleterious effects also accompany reperfusion leading to a depression in cardiac function (myocardial and endothelial).

When oxygen supply to the heart is reduced several changes occurs to both the endothelium and myocardium, which set in play the resultant injury. First, with decreased blood supply the myocardium and endothelium quickly depletes its adenosine triphosphate (ATP) stores and its energy substrates (glucose and triglycerides). As ischemia continues anaerobic metabolism to maintain energy demands begins. The consequences of anaerobic metabolism include decrease in intra and extra cellular pH, decreased contractility, and a decrease in the membrane potential of the myocyte while resulting in endothelial dysfunction. As lactate starts to accumulate and pH decreases further, there begins an accumulation of osmotically active particles such as inorganic phosphates. This increase in
intracellular osmolarity causes an increase in water content, which further reduces cardiac function by decreasing compliance and impairing vasomotor function. NO synthesis is also decreased during ischemia, which results in a slight decrease in contractility as well as impaired vasodilation and increased sensitivity to vasospasm [12;126-130]. But the major effect of NO’s decrease is a loss of its vasodilatory effect, which results in decreased collateral vasodilatation, increased neutrophil activation and macrophage activation [127-135]. This activation results in the production of superoxide and other free radicals, which cause a decrease in endothelial and myocardial function [136-138]. Furthermore, NO combines with superoxides to produce peroxynitrite (PNT) [139]. Neutrophils and macrophages are activated by free radicals produced during ischemia and by the decreased levels of NO [135]. Activated leukocytes then secrete cytokines, which potentiate more free radical production and stimulate both eNOS and iNOS [135].

PNT is commonly known as a very potent free radical that is produced by the reaction of NO with superoxide [139]. There are complex reactions between PNT and endothelium, endocardium and cardiomyocytes that result in either beneficial or harmful effects depending on substrate availability or the biologic environment. PNT in low concentrations at basal state can act as a detoxifier. This detoxifying role occurs at basal state by the reaction between PNT and thiol-containing substances. This reaction leads to the formation of nitrosothiols which potentially detoxifies the anion. Therefore, PNT is detoxified and hence one mole of superoxide is also detoxified. The mechanism of injury by PNT is by hydroxylating and nitrating aromatic compounds. Hence, cellular injury is induced by lipid peroxidation, DNA fragmentation, damage to proteins and plasma lipids and depletion of antioxidants such as glutathione and nitrating protein [140-148]. This injury
is produced by a very reactive intermediate of PNT ONOOH- [148]. PNT can also result in injury by reacting with carbon dioxide leading to the formation of free radicals, which eventually results in the accumulation of nitronium [145]. Nitronium nitrates proteins such as cardiac proteins which leads to impaired cardiac function such as decrease contractility and endothelial dysfunction [145].

Several investigators have studied the effect of PNT on the heart during I/R [149-153]. PNT can inhibit cardiac function by inhibiting oxidative metabolism, which leads to decreased inotropic force and impaired vasodilation [152;153]. Other mechanisms include increasing intramyocardial water content as a result of increased vascular permeability, resulting in increased ventricular stiffness and therefore decreased diastolic function [150;152]. In higher concentrations, PNT can cause cellular necrosis leading to cardiac dysfunction (myocardial and endothelial). PNT is known to cause significant endothelial dysfunction post reperfusion by decreasing the response to vasodilators, by direct endothelial damage and increased vascular permeability [151]. These mechanisms all contribute to decreased blood supply to the myocardium and lead to reduced myocardial oxygen supply thus inhibiting oxidative metabolism and impairing cardiac function.

Another change seen in ischemia is the stimulation of myocardial eNOS. However, during ischemia there is a lack of substrate availability and a change in the intracellular milieu resulting in the uncoupling of the enzyme. In this uncoupled state eNOS produces superoxide radicals, which results in increased PNT production, further activation of leukocytes, decreased NO availability, decreased cardiac function, and further production of free radicals therefore forming a vicious cycle. Furthermore, iNOS transcription is stimulated but acutely there are low levels of iNOS. This low level of iNOS produces
superoxide, which increases the damage to both the endothelium and the myocardium and causes cardiac dysfunction.

Reperfusion of the ischemic myocardium although necessary, may result in worsening of myocardial and endothelial damage. Richard et al demonstrated that for the endothelium the greatest injury occurs during reperfusion [154]. There is a loss of the ability to release NO within minutes following reperfusion [14;21;25;126-130;135;138;154-160]. This loss is related to the consumption of NO by superoxide radicals formed early during reperfusion [136;137]. During reperfusion there is a sudden burst of oxygen delivered to the ischemic zone. This increase in oxygen tension results in a drastic increase in superoxide anion produced both by eNOS and iNOS. The sources of the superoxide radicals are the myocardium, endothelium and leukocytes. Free radical production is also increased, as is PNT production. This massive accumulation of reactive species results in depression of myocardial contractile function, decrease in myocardial compliance, endothelial dysfunction and decreased NO bioavailability [127-130;135;138;154;156-160]. Furthermore, there is a reduction in blood flow to the affected area as a result of decreased NO production, injured endothelium, and increased platelet aggregation, which results in further cardiac dysfunction [127-130]. Reactive oxygen species also activate and stimulate cytokine production, which stimulates further free radical production by enhancing eNOS and iNOS activity. In addition, endothelial cells become activated and cell surface endothelial adhesion molecules are expressed which promote the adhesion of circulating leukocytes [12;79;80;135]. Activated endothelial cells produce and release cytokines (such as ET-1), which promote the adhesion of circulating leukocytes and ROS generation [161;162].
ET-1 is a cytokine released by activated endothelial cells. ET-1 is one of the most potent endogenous vasoconstrictors and mediates a host of responses including endothelial dysfunction, vasomotor contraction, leukocyte activation and cellular proliferation. Additionally, it augments the vascular actions of other vasoactive substances such as angiotensin-II, norepinephrine and serotonin. In the endothelial cell, ET-1 is produced by preproET under the influence of the ECE. ET-1 exerts its biological effects via binding and activating ET-1 receptors (ET\textsubscript{A} and ET\textsubscript{B}), with the former mediating the bulk of the vascular actions. Diminished production of NO and exaggerated release of ET-1 are believed to be key initiators of endothelial injury. ET-1 also results in leukocyte activation and production of ROS. We have previously demonstrated in our laboratory that attenuating the release of ET-1 following I/R can enhance cardiomyocyte tolerance to I/R [15;16]. We have previously shown in a human cardiomyocyte cell model of simulated I/R that ET-1 antagonism improves cell survival [16]. Furthermore, elevated ET-1 levels worsen I/R injury and results in impaired vascular function as assessed by videomicroscopy of atrial arterioles [16]. In addition, ET-1 antagonism attenuated ischemic injury and improved vasomotor function [16]. BOS is a potent ET-1 receptor antagonist and may prove beneficial in endothelial protection. In previous cellular studies, we demonstrated that BOS exposure reduced cardiomyocyte ET-1 production leading to improved tolerance to ischemia [15].

Several investigators have demonstrated that ET-1 alters protein kinase C (PKC) PKC and Akt/PKB activity [163-165]. Furthermore, in an I/R model, Mochly-Rosen et al. have demonstrated that either cardioprotection or injury occurs depending on the PKC isoform that is activated [166-168]. They demonstrated that PKC\textepsilon activation and PKC\textdelta
inhibition is cardioprotective [166-168]. Therefore, determining the role of PKC in endothelial injury during cardiac transplantation can lead to the development of pharmacological therapies to reduce I/R injury at the time of transplantation and the development of CAV. In addition PKC activity can modulate NO production and affects caveolin-1 interaction with cytosolic proteins. The role of PKC and ET-1 in transplant induced endothelial injury is not fully understood and is the subject of these investigations.

In summary, NO levels are decreased in I/R with concurrent increases in superoxide and PNT production resulting in significant impairment of both endothelial and cardiac function. Furthermore, ET-1 release is increased, which leads to vasoconstriction, ROS generation and leukocyte activation.

1.3 PRECONDITIONING (PC) (Figure 3)

1.3.1 Ischemic Preconditioning

In 1986, Murry et al., first described ischemic preconditioning (IPC) [169]. There have been several studies describing this phenomenon [170-172]. There is an early phase lasting for a few minutes to several hours and a late phase, which starts after 12 hours and last up to three days. The phenomenon of IPC occurs in several species (mice, rats, pigs, dogs, and rabbits) and has been described in both human endothelial cells and cardiomyocytes [169-183]. IPC is achieved by generating several short periods of ischemia followed by reperfusion for at least one minute [184]. There is some controversy on the whether IPC is an all or nothing phenomenon or whether IPC induces a graded response. Several investigators found that too few or too many cycles of I/R had no effect on the degree of protection afforded by IPC, whereas; others found that the level of myocardial
protection varied directly with the intensity of the IPC stimulus [185-187]. Endothelial IPC has also been studied [127;154]. Richard et al demonstrated that IPC can prevent coronary endothelial dysfunction [154].

1.3.2 Pharmacologic Preconditioning

Since the description of IPC by Murry et al. several studies investigated the mechanisms behind its protective effects. Determining the mechanisms by which IPC confers myocardial preservation may eventually lead to the development of therapies to reduce cardiomyocyte injury following cardiopulmonary bypass. These studies led to the discovery that PC can be induced by pharmacological means [188-198]. The protective effects of pharmacologic PC (66-85% reduction in infarct size) are similar to that observed in Murry’s original study (75% reduction in infarct size) [199-209]. In addition, the cardiac surgeon can utilize pharmacologic PC readily through direct access via the cardioplegic cannulae. Pharmacologic PC offers the promise of an easy and effective way to mimic PC without ischemia and may provide additional benefit to cardioplegic protection. The theoretic enhanced protection of pharmacologic PC over IPC may occur through the simultaneous effects of cardioplegia (hypothermia and cardiac standstill) and the PC mimetic versus the protective effect of IPC followed by the protective effect of cardioplegia as seen with current IPC strategy. However, clinical studies demonstrate conflicting results. Teoh et al. demonstrated the IPC was superior to both cardioplegia and pharmacologic PC whereas Kevelaitis et al. showed that pharmacologic PC did offer myocardial preservation and that it enhanced the protective effects of IPC [210-213]. Current studies principally focus on the effects of PC on the myocardium and not the endothelial cell. Several investigators have demonstrated that endothelial cell can become preconditioned [173-182].
Ma et al showed that pharmacologic PC can be induced on endothelial cells [174]. They demonstrated that lipoteichoic acid can results in endothelial cell PC. In addition they revealed that this effects was mediated by NO [174]. Kaeffer et al found that PC can prevent coronary endothelial dysfunction [127]. Compared to the myocytes, endothelial cell pharmacologic PC is less well studied. However, pharmacologic PC offers an excellent opportunity to protect the endothelium from a future ischemic insult.

1.3.3 Post Preconditioning

Postconditioning is the event that renders protection following the ischemic insult either pharmacologically or ischemically. Postconditioning, brief periods of ischemia performed just at the time of reperfusion, can be easily performed at the end of the prolonged ischemic insult. Zhao et al. first described this phenomenon [214]. Using a canine left anterior descending artery (LAD) ligation model they compared the protective effects of IPC to that of postconditioning. Their control groups received an LAD occlusion for 60 minutes and reperfused for 3 hours, the IPC group had the LAD occluded for 5 minutes followed by 10 minutes of reperfusion before regional ischemia and the postconditioning group had reperfusion initiated for 30 seconds followed by 30 seconds of reocclusion which was repeated for three cycles after LAD occlusion. Their study showed that postconditioning reduced infarct size by 44% compared to control while no differences were seen compared to IPC [215]. Zhao’s observations indicated that postconditioning preserved the myocardium to the same extent as IPC [216]. They further demonstrated that the beneficial effect of IPC and postconditioning was not related to improved collateral flow [217]. In addition they showed that plasma creatine kinase (CK) and tissue myeloperoxidase activity were lower in the IPC and postconditioning groups [218]. One of the most important findings of their
study was that postconditioning offered the same protection as IPC and that endothelial function as assessed by the vasodilator responses to Ach was improved by postconditioning [219]. Following this manuscript Kin et al. demonstrated the benefit of postconditioning in a rodent model [220]. They showed a 23% reduction in infarct size however this benefit was significantly lower than IPC which resulted in a 56% reduction [221]. These studies indicate that there may be species variability in the effectiveness of postconditioning. Tsang et al. observed the myocardial protective effects of postconditioning with a 38% infarct size reduction [222]. They showed similar benefit in infarct size reduction between IPC and postconditioning [223]. Their study also addressed the issue of combining IPC with postconditioning and showed no added benefit [224]. Halkos et al., in a canine model confirmed Tsang’s findings that IPC had no additive effect on postconditioning protection [225]. The only clinical study on postconditioning is that of Staat et al. [226]. Their study was performed in patients undergoing angioplasty for ongoing acute myocardial infarction [227]. After stenting the postconditioning group had four episodes of 1-minute inflation and 1-minute deflation of the angioplasty balloon. This protocol had a 36% reduction in serum CK levels compared to the control group [228]. Their data showed that postconditioning could be applied clinically. This study demonstrated one of the advantages of postconditioning over IPC in that it can be initiated after the ischemic insult has occurred. Unlike PC, postconditioning does not require initiation prior to the ischemic event. The cardiac transplant surgeon can also benefit from postconditioning. The storage time of the cardiac allograft can often exceed the protective effects of early PC and therefore, postconditioning may play an important role for allograft protection following storage and transplantation. The optimal postconditioning strategy would be pharmacologic.
Pharmacologic postconditioning would avoid the adverse consequences associated with intermittent cross-clamping and provide a simple method of cardiac protection following all cardiac procedures. Pharmacologic postconditioning in endothelial cell is not well studied. Pharmacologic pre/post conditioning offers interesting avenues to pursue in protecting the endothelial cell from hypoxic injury. Another possible strategy is to treat the endothelium during the ischemic/hypoxic insult to limit the injury seen with reperfusion.

1.4 NITRIC OXIDE REGULATION

1.4.1 Nitric Oxide

NO plays an important physiologic role in the cardiovascular system. It has basal functions, regulatory functions, and potent responses to various stimuli. NO plays a critical role in vasomotor control but in the past decade, NO has been shown to play a role in both I/R injury and in IPC. Therefore, understanding the physiology of NO in basal states and during I/R may allow for the development of methods that can limit I/R injury and confer the PC effect pharmaceutically. Chemically, NO is lipophilic and a highly reactive unstable gaseous molecule. The sources of NO can be endogenous or exogenous. NO is involved in regulating vascular (vasomotor) tone, helps maintain a nonthrombogenic endothelial surface, inhibits platelet aggregation, decreases leukocyte-endothelial cell interaction, and inhibits vascular SMC proliferation [133;229-233]. NO also is a neurotransmitter of the central nervous system [234]. The above functions of NO underline its importance in maintaining normal homeostasis. NO may also have detrimental effects depending on the circumstances. Such effects include hypotension in endotoxemia, activation of macrophages, and production of ROS.
Endogenous sources of NO that affect the heart are from the endothelium, the SMC and the myocardium [235]. NO has endothelial protective effects, it maintains vascular homeostasis, reduces platelet aggregation, decreases leukocyte adhesion and activation [133;229-233] and maintains vascular homeostasis by inducing SMC relaxation. SMC relaxation is achieved by a cyclic guanosine monophosphate (cGMP)-dependent pathway [236-257]. The importance of eNOS and NO in vascular homeostasis was demonstrated by Huang et al who found that mice lacking the eNOS gene had hypertension and displayed an inability of the endothelium to respond to vasodilatory stimuli [243]. NO released by the endothelium and endogenous SMC derived NO causes the activation of guanylyl cyclase which in turn results in cGMP accumulation [237;241;244;246;254]. cGMP accumulation activates protein kinase G [237;241;244-246;249;251;254;255;257]. PKG has principally three effects on SMC leading to vasodilation [236-257]. PKG phosphorylates potassium channels resulting in SMC hyperpolarization and therefore decreased Ca\(^{2+}\) entry and vasodilation [253;254;256]. PKG phosphorylates an inositol-1,4,5 triphosphate (IP3)-receptor associated cGMP kinase substrate which decreases Ca\(^{2+}\) release from the sarcoplasmic reticulum [246;247;250]. PKG also phosphorylates phospholamban which causes inhibition of sarcoplasmic reticulum ATPase inhibition causing Ca\(^{2+}\) sequestration and vasodilation [246;247;250;258]. Decreases in intracellular Ca\(^{2+}\) results in decreased formation of myosin light chain kinase complex which decreases contraction [244;254;257]. cGMP also inhibits cAMP-PDE leading to cyclic adenosine monophosphate (cAMP) accumulation and activation of protein kinase A (PKA) [248;259-262]. PKA activation leads to potassium channel activation and decreased Ca\(^{2+}\) entry directly and can also increase PKG activity further increasing the cGMP response [248;259-262].
Two different mechanisms, a cGMP-dependent and a cGMP-independent pathway mediate NO action on the myocardium regardless of the source of NO [263;264]. It has been known for several years that NO affects contractility but the direction of the effect was not completely understood. In some studies NO increased contractility while in others NO decreased contractility [264-271]. Accounting for the concentration of NO used to elicit the effect on the myocardium clarifies the role of NO on basal cardiac function and during states of increased NO production. At basal states [0.05\(\mu\)m], NO causes a positive inotropic effect on the myocardium [264;268-270]. Whereas at higher concentrations [10\(\mu\)m+] NO exerts a negative inotropic effect [265-267;271]. The mechanism of this opposing effect occurs through the action of cGMP. At basal concentrations, NO causes the activation of GC, which leads to the production of cGMP. This elevation of cGMP then causes the inhibition of cAMP-phosphodiesterase (PDE), which leads to the buildup of cAMP. Accumulation of cAMP results in the activation of cAMP-dependent PKA, which increases Ca\(^{2+}\) entry via L-type channels. This eventually causes increased contractility. As the concentration of NO rises beyond the basal state levels there is a further increase in cGMP levels, which causes the activation of cGMP-dependent protein kinase (PKG) [272] resulting in inhibition of Ca\(^{2+}\) entry into the cell via the L-type channels. Furthermore, at higher concentrations NO can directly inhibit the L-type Ca\(^{2+}\) channels. Therefore, higher concentrations of NO cause a negative inotropic effect. NO also has been shown to effect lusitropy although the mechanism is not fully understood and may be mediated by PKG [273;274]. Under experimental models NO seems to result in a positive chronotropic effect that is caused by activation of the hyperpolarization-activated inward current [275]. This effect is seen in
cardiac transplant patients given nitrate but how important this mechanism is in a normally beating heart is unclear.

1.4.2 Nitric Oxide Synthase

NOS (135 KDa) is the enzyme that produces NO in vivo [134]. NOS is a family of enzymes, located on chromosome 7, which share several functions in common but also differ in many ways. NOS catalyses the formation of NO by converting L-arginine to L-citrulline. This biosynthesis of NO occurs when NOS is in a dimeric form composed of two identical monomers. The enzyme is divided into two functional domains 1) the C-terminal reductase domain and the N-terminal oxygenase domain. The C-terminal domain houses the binding sites for one molecule of FAD, FMN, and nicotinamide adenine dinucleotide phosphate (NADPH). The N-terminal domain binds heme, tetrahydrobiopterin (BH₄), and L-arginine. Furthermore, there is a calmodulin-binding domain, which lies between both the C- and N-terminal domains. This calmodulin-binding domain has an important role in maintaining enzyme structure and function.

The NOS family consists of three different isoforms [276], which differ not only in their structure but also in their function. These three enzymes are neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS). The major differences between these enzymes are that both eNOS and nNOS are expressed constitutively and that they are Ca²⁺-dependent enzymes whereas, iNOS expression requires induction and its function is Ca²⁺ independent. All three of these isoforms have regions of homology in both the reductase and oxygenase domain but it is in regions in which they differ that confers their enzyme specific function.
The formation of NO occurs in two steps (Figure 4). First, NOS oxidizes L-arginine into N-hydroxy-L-arginine. Then, it oxidizes N-hydroxy-L-arginine into L-citrulline and NO. The first reaction requires the consumption of one mole of oxygen and one mole of NAPDH. The second step requires the consumption of one mole of oxygen and a half mole of NADPH. This reaction only occurs when the enzyme is in its dimeric form [277;278]. Studies have shown that the reductase domain also plays a key role in the synthesis of NO [279;280]. It is the reductase domain that allows electrons to be transferred from NADPH to FAD and FMN to cytochrome c. This electron transfer chain allows the oxygenase domain to convert L-arginine to L-citrulline and NO. Therefore, the biosynthesis of NO requires both domains to perform different functions simultaneously. The formation of NO requires the enzyme to be in the dimeric form to allow the transfer of one electron from one reductase domain subunit to the oxygenase domain of the other subunit. Hence, the function of NOS is not only related to its catalytic domains but to its quaternary structure.

NOS synthase catalytic activity is not limited to the synthesis of NO. NOS can synthesize superoxide and PNT. The formation of superoxide occurs when the NOS enzyme is in the monomer form. As a monomer NOS is unable to produce any NO. PNT can be formed when superoxide and NO are in close proximity. States that cause NOS to be in the monomeric form such as low BH$_4$ concentration, low L-arginine availability, or I/R result in increased production of free radicals and decreased production and bioavailability of NO [281;282].

Factors that regulate homodimerization will be discussed in brief. There are three such essential factors; heme, L-arginine, and BH$_4$. First, the heme moiety plays an important role in homodimerization. In the absence of heme none of the NOS isoforms can exist in the
dimer form. Furthermore, the heme moiety is the only factor that is absolutely required for the generation of an active dimer form. As mentioned above, the electron transfer occurs between monomers and not within monomers during the biosynthesis of NO. This quaternary structure has the reductase domain of one subunit opposing the oxygenase subunit of the other and is facilitated by the presence of the heme moiety. L-arginine plays an important role in NOS dimerization even though it is not absolutely required. The binding of L-arginine causes conformational changes in the NOS structure allowing for a more stable quaternary form, which causes the close alignment of the heme unit to BH$_4$. The function of BH$_4$ in dimerization has been characterized. BH$_4$ is not absolutely required for the formation of the dimeric form but BH$_4$ is an absolute cofactor required for the activation of eNOS and production of NO [283-288]. Mechanistically, BH$_4$ functions as a reducing cofactor, transferring electrons to the enzyme bound L-arginine. BH$_4$ promotes stabilization of the dimeric (active) form of NOS and also increases the affinity of NOS for L-arginine. In the presence of low levels of BH$_4$, NOS functions as an NADPH oxidase resulting in the production of oxygen derived free radicals instead of NO. In other words, eNOS is uncoupled in the presence of low levels of BH$_4$ with the resultant production of superoxide anions vs. NO. BH$_4$ also acts as a free radical scavenger, which can limit NO radical toxicity and superoxide damage.

Ca$^{2+}$ has been found to play a critical role in NO formation. First, Ca$^{2+}$ is required only for the NO producing function of nNOS and eNOS. Ca$^{2+}$ binding to the calmodulin domain of both the nNOS and eNOS isoforms causes activation of the enzyme and formation of NO [289]. The iNOS isomer binds calmodulin irreversibly and therefore, its activation is Ca$^{2+}$ independent [290]. Calmodulin binds to the calmodulin-binding region of
NOS. This binding region also causes NOS to be associated with plasma membranes. When associated with plasma membranes the enzyme is less active or inactive. Once the Ca\(^{2+}\) concentration rises in the cell and binds to the calmodulin-binding region, NOS dissociates from the membrane and becomes active [291]. Calmodulin is also involved in the electron transfer between reductase and oxygenase domains [292].

Endothelial cells express both eNOS constitutively and iNOS when induced but do not express any detectable level of nNOS. Examining eNOS and iNOS more closely reveals differences between both enzymes. First, eNOS is constitutively expressed in both coronary endothelium and in cardiomyocytes especially in the endocardium. eNOS has one property that the other isoforms does not possess, which is its ability to respond to hemodynamic stresses. Even though eNOS is constitutively expressed, its expression can be enhanced and its activity increased by several mechanisms including exercise and shear stress. eNOS is targeted to specialized invaginations of the plasmalemma termed caveolae [131;293-299]. Plasmalemmal caveolae serve as sites for the sequestration of signaling proteins and are further characterized by the presence of caveolin, an intrinsic membrane protein that forms a structural “scaffold”, organizing both proteins and lipids within this key membrane organelle [131;293-299]. Caveolins (22 KDa) directly interact with several structurally distinct signaling proteins in caveolae, including eNOS. Several groups have independently reported that caveolin-1, the resident integral membrane protein of caveolae, directly interacts with and inhibits eNOS both in-vitro and in-vivo. In the resting state, eNOS is tethered to caveolin-1 and is less active. After provocation by agonists that raise intracellular Ca\(^{2+}\), caveolin dissociates from eNOS resulting in an activated eNOS-calmodulin complex. When the tide of intracellular Ca\(^{2+}\) subsides, the
cycle is reversed [131]. Localization to the plasmalemma is mediated by post-translational modification of eNOS. The enzyme is myristoylated and palmitoylated at its N-terminal site which helps targeting of the enzyme to the plasmalemma. By being localized to the plasma membrane, it allows for close proximity of the enzyme to its molecular signaling pathways, which mediates its activity. eNOS in the basal state is sub-maximally active, due to the Ca\textsuperscript{2+} concentration in the cell at the resting state. Studies have demonstrated that a variety of risk factors including LDL-cholesterol increases the expression of caveolin-1 and the formation of the eNOS-caveolin complex thereby limiting the production of NO. However, the role of ET-1 on the eNOS-caveolin interaction is not fully defined in human endothelial cells. Other than Ca\textsuperscript{2+}, enzyme activity can be promoted by several hormones such as catecholamines, bradykinins, and ADP. Substrate and cofactor availability, especially L-arginine and BH\textsubscript{4}, have an impact on enzyme activity. Therefore, the pathways that generate L-arginine and BH\textsubscript{4} play crucial roles in the regulation of eNOS and iNOS activity.

eNOS activity can also be increased by phosphorylation of the serine-1177 residue [300;301]. This phosphorylation occurs under physiological stimulation, such as shear stress, and is mediated by the phosphatidylinositol 3-OH kinase (PI3K) pathway [302]. PI3K phosphorylates and activates Akt kinase, which in turn phosphorylates and activates eNOS [301;302].

iNOS differ from eNOS in three specific ways. First, iNOS is barely expressed constitutively but is induced by several factors such as cytokines [303;304]. The other difference is that the enzyme activity is Ca\textsuperscript{2+} independent. Thirdly, iNOS does not have a membrane targeting sequence. iNOS is a oxygen radical producer mainly through its
reductase domain [305-307]. High rates of ROS production occur when L-arginine is in low supply and iNOS activation can impair eNOS NO production [306].

1.5 PROTEIN KINASES

Signal transduction pathways represent one mechanism by which the cell is able to respond to external stimuli. The extracellular signal (ET-1 for example) binds to a receptor. The activated receptor then directly or indirectly through a transducer (G protein for example) stimulates an effector (such as phospholipids or adenylyl cyclase). The activated effector can then increase the amount of intracellular second messenger (cAMP, DAG, Ca\(^{2+}\)) and/or change second messenger cellular localization. Target proteins are then activated by these second messengers that can directly or indirectly through other target proteins alter protein transcription and translation or enzyme activity.

An important class of target proteins are the serine/threonine kinases. These kinases phosphorylate proteins on either a serine and/or a threonine residue in order to alter activity, cellular distribution or protein breakdown. PKA, PKB and PKC are part of the serine/threonine kinase family. They play a crucial role in signal transduction.

1.5.1 Protein kinase C

Discovered in the late 1970s as a target for tumor-promoting phorbol ester and named in the early 1980s PKC is a large family of serine/threonine kinases (67.2-83.5 kDa) [308-334]. The original description of PKC is that it is a Ca\(^{2+}\) and phospholipid-dependent protein that requires DAG for activation [313-315]. Since this original description three different categories of PKC have been described depending on their requirements for activation. The PKC family consists of at least twelve isoforms of second messenger-dependent protein kinases that are classified as classical, novel or atypical. The classical
isoforms (α, βI, βII, γ) described by Takai et al and Kishimoto et al require Ca\(^{2+}\), phosphatidylserine (PS) and DAG for activation [313-315] (Figure 5). PKC activation occurs when plasma membrane receptors coupled to phospholipase C are activated, releasing DAG and IP3. IP3 then results in Ca\(^{2+}\) release. The novel isoforms (δ, ε, η, θ) require DAG and PS but not Ca\(^{2+}\) for activation [321;335-342]. The third category of PKC isoymes are the atypicals (ι, λ, ζ) which are activated by PS but do not require DAG or Ca\(^{2+}\) [321;322;342-346].

PKC isoymes have an N-terminal regulatory domain (20–70kDa) and a C-terminal catalytic domain (~45kDa) (Figure 6) [330-334]. PKC isoymes contain four conserved regions termed C1–C4. C1 contains the cysteine-rich motif which binds to DAG [325;326;329-334]. Upstream of the cysteine-rich motif lays the autoinhibitory pseudosubstrate sequence [347;348]. The C1 cysteine-rich motif consists of two zinc fingers within six cysteine residues [349]. This domain is present in all PKC isoymes where the classical/novel forms possess two and the atypicals only one [349-355]. This binding domain binds DAG and phorbol esters in DAG sensitive isoymes to form a hydrophobic face allowing membrane targeting [349-355]. For the atypical isoymes, the phorbol ester binding site lacks the consensus residues to form the hydrophobic face [349-355]. The upstream autoinhibitory pseudosubstrate domain of C1 blocks the active site and must be removed to render the isoform active [325;330;347-349;354]. The inhibitory function of the autoinhibitory pseudosubstrate domain comes from its sequence homology (except for an alanine substitution of serine and threonine residues) with the phosphorylation site consensus sequence (xRxxS/TxRx) [325;347-349;352;354;356]. C2 contains the recognition site for acidic lipids and, in some isoymes a β-sheet domain containing the Ca\(^{2+}\) binding
site [325;347-349;352;354;356-361]. The binding of Ca$^{2+}$ results in a conformational change leading to exposure of lysine residues resulting in increased affinity for PS [325;347-349;352;354;356-364]. The Ca$^{2+}$ insensitive isoforms do not require Ca$^{2+}$ for increased binding to PS [325;347-349;352;354;356-358;362-364]. C1 and C2 together compose the regulatory domain. C3 and C4 form the ATP and substrate binding lobes of the kinase, respectively. [325;325-327;329;330;330;342;347-350;352;354;354;362-365] The C3 domain is relatively well conserved except for the zeta isoform which has a single amino acid substitution [325;327;330;342;354;362-371]. The C4 domain contains both the substrate binding and the phosphorylation site [325;327;330;342;354;362-371]. C3 and C4 form the catalytic domain.

PKC requires activation to initiate its kinase activity except for PKC$\zeta$ which has a low baseline activity without any external activation due to amino acid substitutions in its C3 and C4 domains [309;313-316;327;328;330;331;352;354;360;361;372-374]. In the inactive form, the pseudosubstrate domain is bound to the catalytic domain of PKC and the kinase is located in the cytosol [309;313-316;327;328;330;331;347;348;352;354-356;362;370;372-374]. Inactive PKC is not freely distributed throughout the cytoplasm but appears localized to specific sites within the cell. This localization is facilitated by association of PKC with scaffolding proteins, such as A Kinase-Anchorin Protein 79 (AKAP79) and Gravin [375-380]. AKAP79 binds a number of PKC isoforms including $\alpha$, $\beta$II, $\delta$, $\epsilon$, and $\zeta$ [375-380]. Activation of the PKC isozyme requires removal of the pseudosubstrate from the active site [348;352;354-356;362;370]. Removal of the pseudosubstrate occurs through a conformational change of both C1 and C2 domains [309;313-316;320;327;328;330;331;347;348;350;352;354-356;358;362;370;372-374;381].
DAG is produced from the hydrolysis of phosphatidyl 4,5-inositol biphosphate (PIP2) to produce IP3 and DAG [309;313-316;320;327;330;347;348;350;352;354;355;358;360-362;370;372-374;381]. DAG then binds to the C1 domain which can translocate the enzyme to the membrane with low-affinity. High-affinity translocation and binding occurs when PS is also bound to the C2 domain [382]. When both PS and DAG are bound to the classic isozymes the pseudosubstrate can be removed from the active site. PS binding to the classical isozymes requires Ca$^{2+}$. PS acts more as a cofactor in classical PKC activation as it increases DAG binding which in turn activates PKC. Ca$^{2+}$ is not absolutely required for activation but lowers the concentration of phorbol ester required to activation. DAG binding indirectly increases the isozyme affinity for DAG by increasing the C2 domain specificity for PS. When C1 in unbound the C2 domain can bind any acidic phospholipid head however, when DAG is present C2 specificity is for PS which in turn increases DAG binding and hence activation. For the novel isozymes Ca$^{2+}$ binding is not required for activation as the C2 domain has an amino acid substitution of an aspartate for an arginine which results in a C2 conformation that is always receptive to PS binding [314;327;330;340;341;347;348;350;352;354;355;357;360-362;370;372-374;381]. The atypical isozymes do not require DAG or Ca$^{2+}$ for activation. PS binding is sufficient to result in PKCζ, λ and ι activation [327;330;340;341;347;348;350;352;354;355;360-362;372-374]. Other lipids have been demonstrated to result in PKC activation such as arachidonic acid and polyphosphoinositides. PKC binding proteins have been identified that localize activated PKC [383-393]. The Receptor Associated with C Kinase (RACK) binds only activated PKC and directs it to the appropriate subcellular site [383-393]. Specific
RACKs bind to isoform specific PKCs which help in the isoform-specific function of the kinase [383-393].

Phosphorylation and autophosphorylation of PKC plays an important role in PKC activation [325;365;369]. Three phosphorylation sites are present on PKC [325;363;365;369;394-397]. One site is located in the activation loop [325;363;365;369;394-397]. The importance of phosphorylation of the activating loop was demonstrated by Cazaubon et al and Orr et al who showed that a mutation of the threonine residue to a nonphosphorable residue resulted in inactivity [363;395;396]. They showed that phosphorylation is required for proper alignment of the activating loop residues [363;395;396]. PKC is unable to autophosphorylate this site therefore requires another kinase for phosphorylation. One such kinase is phosphoinositide dependent kinase-1 (PDK1) [398-402]. The activating loop is also masked when the pseudosubstrate is located in the active site. Therefore, PKC must be bound to DAG and/or PS to expose the activating loop for phosphorylation. Once the activating loop is phosphorylated autophosphorylation can occur at its carboxy-terminal also known as the turn motif and hydrophobic motif [325;363;365;369;394-397]. Autophosphorylation occurs in all PKC isoymes [325;363;365;369;394-397;403;404]. The importance of this step is to lock the enzyme in an active competent state. A third autophosphorylation site is located on serine660 which assists in subcellular localization. Other phosphorylation sites exist which can regulate activity [405;406]. Therefore, activation of PKC requires a conformational change of the regulatory domain by DAG and/or PS and/or Ca\(^{2+}\), followed by phosphorylation of the activating loop then autophosphorylation followed by ATP binding to the catalytic domain in addition to interacting with the appropriate RACK prior to C4 binding its substrate.
Composition of PKC isoforms with each tissue/cell type varies. The brain possesses all isoforms whereas the other cell types have various isoform profiles. In addition, PKC isoform activation and inhibition is cell type and stimulus specific. A given cell may react to a stimulus by activating one isoform, inhibiting another and not affecting the remaining isoforms. The same stimulus in another cell type may have no effect, the same effect or even opposing effects. The downstream effects of activation are also isoform and cell type specific. Therefore, the effects of PKC activation are dependent on the stimulus and the cell type. Chen et al demonstrated that PKCδ and PKCε can have opposite effects and parallel effects in the same cell type. They found that PKCδ activation and PKCε inhibition causes cell death whereas PKCδ and PKCε activation causes hypertrophy [407].

PKC plays a pivotal role in mediating cellular responses to extracellular stimuli involved in proliferation, differentiation and apoptosis. PKC functions as the transducer of a second messenger (Ca^{2+} and DAG). PKC is also involved in receptor desensitization, in modulating membrane structure events and in regulating transcription. PKC plays an important role in normal vessel homeostasis, I/R, NO and ET-1 regulation, IPC and cell death. The first role ascribed to PKC is its ability to alter gene expression [320]. Kikkawa et al demonstrated that PKC was the receptor responsible for tumor-promoting phorbol esters [320]. Another important function of PKC is its ability to alter receptor sensitivity [408]. Bollag et al showed using an in vitro model that PKC can phosphorylate the insulin receptor and lower its kinase activity [408]. The ability of PKC to alter proliferation was demonstrated by Donson et al [409]. In a human glioblastoma cell line they observed that PKCζ activation was responsible for proliferation [409]. PKCζ has also been demonstrated to be involved in neutrophil adhesion and chemotaxis [410].
Braun et al found isoforms can have opposite effects [411]. They observed that PKCζ stimulates fibroblast proliferation whereas PKCδ inhibits [411]. Gliki et al showed that PKC has the ability to alter other serine/threonine kinase activity [412]. They demonstrated that PKC can modulate Akt/PKB activation and vascular endothelial cell growth factor (VEGF)-stimulated angiogenesis [412]. Their data revealed that PKC can regulate angiogenesis [412]. PKC also has important effects on basal function [413]. Harrington et al using an endothelial cell culture model showed that PKCδ plays a crucial role in maintaining endothelial cell barrier function [413]. Beckman et al found that in endothelial cells PKCβ can prevent endothelial dysfunction caused by hyperglycemia [414]. They also hypothesized that this was achieved by PKCδ stabilization of microfilamentous structures and by regulation of RhoA GTPase activity [413]. Zhou et al showed that PKC can mediate actin disassembly in mesangial cells exposed to high glucose concentrations mimicking diabetes [415]. Therefore, PKC not only regulates cellular function to external stimuli but is also involved in normal cellular homeostasis.

As mentioned above, PKC plays a role in I/R and in IPC [416-421]. Armstrong et al observed that PKC inhibition can protect rabbit cardiomyocytes from an ischemic insult [416]. Mochly-Rosen et al demonstrated that PKCε inhibition and PKCδ activation leads to cell death [166-168;407]. Baines showed that PKCε activation results in Bad phosphorylation and inactivation leading to myocardial protection [417]. Bright et al found that PKCδ activation during reperfusion mediates cerebral ischemic cell death [420]. Apoptosis is also modulated by PKC [338;422-424]. The effects of PKC on apoptosis are conflicting with some studies demonstrating PKC activation is pro-apoptotic while others showing anti-apoptotic effects [424]. These findings indicate that
isoform specific regulation occurs. Leszczynski et al revealed that PKC inhibition has pro-apoptotic effects leading to Bcl-2 expression in vascular smooth muscle cell [425]. PKCε has also been shown to play a role in apoptosis [422;426]. Okhrimenko et al demonstrated in glioma cells that PKCε regulates apoptosis [422]. Shizukuda et al showed that PKCδ is involved in hyperglycemia induced cardiomyocyte apoptosis [423]. PKC regulation of apoptosis is isoform specific and cell type specific.

PKC can also modulate NO and ROS production [414;427-432]. The general assumption that PKC inhibition increases NO and that PKC activation decreases NO has been challenged. It is now believed that isoform specific changes in specific cell types result in either eNOS activation or inhibition [414;427-432]. Partovian’s study challenged that general assumption and demonstrated that PKCα activation results in NO production. Beckman et al showed that PKCβ inhibition results in NO release [414;430]. In rat endothelial cells, Bohlen et al observed a PKCβ inhibitory effect on NO production [433]. PKC modulates eNOS activity by altering eNOS serine and/or threonine phosphorylation [414;427-432]. Changes in eNOS phosphorylation alters its activity (reduces, increases or inactivates) [414;427-432].

PKC activity can also modulate ROS production. Jeon et al demonstrated that PKCδ activation can lead to ROS production in pancreatic cells [434]. Shizukuda et al found the PKCδ increases ROS in ventricular myocytes [423]. Kwan et al revealed that PKCζ increases mesangial cell free radical production [435]. Xia et al demonstrated that the atypical PKCζ isozyme can increase ROS production from mesangial cells by upregulating NADPH oxidase [432]. PKC can also indirectly activate eNOS through the activation of another serine/threonine kinase [436]. Naruse found that PKC can activate
eNOS NO production via Akt/PKB phosphorylation of eNOS [436].

The role of PKC in human endothelial cell NO and ROS production remains to be fully elucidated. It remains controversial which isozymes are involved and their specific effects on eNOS. The PKC isozyme profile that occurs after human endothelial cell hypoxia also remains to be understood.

1.5.2 Protein kinase A

cAMP-dependent protein kinase is a serine/threonine kinase [437-446]. Discovered and named in the mid-late 1960’s through studies on glucose metabolism [437-446], cAMP-dependent protein kinase is also known as PKA. The kinase is composed of two subunits, a regulatory and a catalytic subunit (Figure 7) [437;438;445-460]. In the inactive state, PKA is in a tetrameric or dimeric form [437;438;445-460]. In this configuration the regulatory subunit inhibits the catalytic subunit [437;438;445-460]. Activation occurs when two cAMP molecules bind to the regulatory subunit and decreases the affinity of the regulatory subunit for the catalytic subunit (Figure 8) [437;438;445-465]. The dissociation of the regulatory subunit (dimeric form) exposes the catalytic subunit (two monomeric forms) and results in PKA activation [437;438;445-460]. In the monomeric form (catalytic subunit), the kinase is constitutively active as the catalytic unit does not require activation for activity. The catalytic subunit can also be inhibited by a protein kinase peptide inhibitor (PKIP) [466-469]. The PKIP acts as a pseudopeptide which prevents the catalytic function of PKA [466-469].

The regulatory subunit has the ability to dimerize at its N-terminus [437;438;446;451-460;470-473]. The regulatory subunit is composed of several isozymes that form homodimers but heterodimers also occur [437;438;446;451-460;470;471]. Each
The isozyme has relatively conserved functional domains but differ in their N-terminus which confer dimerization ability and confers cellular localization [437;438;446;451-460;470-473]. The C-terminal end contains the autoinhibitory and autophosphorylation region [437;438;446;451-460;470-473]. As with other protein kinases, the autoinhibitory site acts as a pseudosubstrate that inhibits kinase activity. The regulatory subunit possesses two cAMP binding sites [437;438;446;451-460;470-473]. The cAMP binding site is arranged as a tandem repeat [437;438;446;451-460;470-473]. Two cAMP molecules are required for dissociation of the dimer for the catalytic form. [437;438;446;451-460;470-473] cAMP is formed by the conversion of ATP by adenylyl cyclase [461-465;474]. Adenylyl cyclase is activated by G proteins following receptor ligand binding. [461-465;474]

There are several isozymes of the catalytic subunit [448-450]. Therefore, there is the possibility of having several dimers and several isoforms of PKA. All these isoforms differ slightly with cAMP binding affinities that are similar. The main differences may be subcellular localization. The catalytic subunit is a bilobed asymmetric structure [448-450;475-481]. The N-terminus confers the ability to bind magnesium and ATP. The C-terminal lobe provides for peptide recognition and catalysis [448-450;475-481]. The catalytic domain is located in between the two lobes [448-450;475-481]. The catalytic subunit is fully activated in the presence of magnesium and ATP when the regulatory subunit is dissociated [448-450;475-481]. PKA requires magnesium to stabilize the negative charge on ATP. In addition, the binding of the second magnesium molecule increases the binding efficiency for ATP. Therefore, PKA activation requires four cAMP molecules and four magnesium molecules. The peptide recognition domain recognizes the target peptide and transfers one phosphate from ATP to a serine or threonine of the target peptide. The
catalytic subunit also phosphorylates the regulatory subunit at its autophosphorylation/autoinhibitory domain which lowers the affinity for dimerization resulting in increased catalytic monomer formation and hence increased activity. PKA has also displayed the ability to compartmentalize and translocate. This is achieved by the interaction with an anchoring protein [482-488]. A-kinase anchoring protein has been identified to interact with PKA however, not all PKA are bound to A-kinase anchoring protein [482-488].

PKA effects several cellular functions and gene activation. The first role ascribed to PKA was in glycogen and glucose metabolism. Since then PKA has been demonstrated to regulate several cellular processes. PKA has been shown to be involved in hormonal regulation, memory, bone metabolism, ischemia reperfusion injury, PC proliferation and neuronal signaling. [489-497] PKA has an important role in gene transcription since many genes possesses a cAMP-responsive element (CRE) [498-503]. PKA can phosphorylate CRE-binding protein (CREB) and activate transcription of genes that have an upstream CRE [498-503].

Recent evidence suggests that PKA activation may lead to NO production [504]. Hashimoto et al demonstrated that PKA activation can result in NO formation, however, they also found that cilostazol also alters Akt/PKB activation which is known to result in NO production [504]. One possible explanation for Hashimoto’s finding is that PKA activation leads to Akt activation resulting in NO production [504]. Zang et al showed that cAMP induces eNOS activation by promoting Akt/PKB activation in an in vivo canine model [505]. The role that PKA plays in NO production remains unclear. The role of PKA in ROS production also remains to be elucidated. Raha et al showed in rat cardiomyocytes
that PKA can alter NADPH oxidase mediated ROS production [506]. However, Nogueira-Machado et al demonstrated in human granulocytes that PKA activation inhibits NADPH oxidase ROS production [507]. Mitsuyama et al found that PKA inhibition increased ROS production [508]. The role that PKA plays in ROS production remains controversial. PKA is also involved in I/R and IPC [489-493;509-532]. Bolon et al found that I/R reduced interendothelial electrical coupling through a PKA-dependent signaling pathway in mice endothelial cell monolayers [492]. They demonstrated that PKA activation offered protection by maintaining coupling. While Makaula et al showed that PKA inhibition can protect against myocardial infraction in a rodent model, [512] they also demonstrated that PKA activation can act as a PC mimetic [512]. Inserte et al observed that pharmacologic PC with calpain was mediated by PKA [489]. Roninet et al demonstrated in a rat heart model that Akt, PKC and PKA are involved in myocardial pharmacologic PC [511]. Kulhanek-Heinze et al found that PKA is also involved in apoptosis [512]. They revealed that atrial natriuretic peptide induced liver PC was a PKA dependent process resulting in phosphorylation and inactivation of cysteinyll-aspartic-acid-protease 3 (caspase) and BAD [512].

**1.5.3 Akt/Protein Kinase B**

Akt/PKB is a serine/threonine kinase that promotes cellular survival. Akt can be activated by several growth factors such as insulin, IL-3, IL-6, and VEGF. Akt/PKB is composed of three domains: an N-terminal pleckstrin homology (PH) domain, a central kinase domain and a C-terminal regulatory domain which contains the hydrophobic motif. PH domain gets its name from the phosphorylation substrate for PKC in platelets called pleckstrin [533;534]. This domain binds to phosphatidylinositol-1,4,5 triphosphate (PIP3)
and PIP2. The PH domain likely plays a role in Akt’s recognition by upstream kinases such as PKC [533-534]. Downstream of the PH domain rests the central region (kinase domain) of Akt which shares sequence homology with PKA and PKC [535;536]. This region possesses the activation loop which has a conserved threonine residue that requires phosphorylation for activation [535;536]. Adjacent to the activating loops is the hydrophobic motif which also requires phosphorylation to achieve full activation [535;536]. Full activation occurs by phosphorylation of two sites, one in the activation domain and one in the C-terminal hydrophobic motif [535-540].

Akt/PKB becomes activated in a series of steps. First Akt/PKB is translocated to the membrane by the presence of PIP3 [533-541]. PIP3 is formed by activated PI3K [533-541]. PI3K is activated by tyrosine kinases or G-protein-coupled receptor [533-541]. As with PKA and PKC, PKB activity can be modulated by G-protein coupled receptors. Activation occurs by recruitment of PI3K to the receptor on the membrane and then PI3K is activated by PIP2 [533-541]. PIP2 is the same second messenger that can activate PKC. As mentioned above, Akt is activated by a sequence of phosphorylation. Akt phosphorylation of the activation domain can occur by PDK1 [533-541]. Akt activation may also be achieved through PI3K independent means, by phosphorylation of Akt by kinases such as PKA [542;543]. PDK1 phosphorylates threonine$^{308}$ in the activating loop [533-541]. This phosphorylation partially activates Akt/PKB. Full activation requires phosphorylation of serine$^{473}$ in the regulatory tail. PDK1 is also involved in serine$^{473}$ phosphorylation but other kinases have been implicated. The regulation of serine$^{473}$ phosphorylation is not fully elucidated and may involve PDK2 or integrin-linked kinase [533-541].
Akt is principally a cell survival kinase that regulates apoptosis, gene transcription and cell cycle. It exerts anti-apoptotic effects through phosphorylation of substrates that directly regulate the apoptotic machinery such as Bad or caspase 9, or phosphorylation of substrates that indirectly inhibit apoptosis such as forkhead (FOX) transcription family members, or NF-κB kinases [544-561]. Akt phosphorylates and therefore inactivates BAD. Phosphorylated BAD is translocated out of the mitochondria and in the cytosol inactivates caspase 3 [544-549]. Ohi et al demonstrated that maintenance of BAD phosphorylation prevents sinusoidal endothelial cells apoptosis [550]. Nishimura et al found in vascular endothelial cells that Akt plays an important role in promoting survival [551]. They showed that endothelial cells transfected with inactive Akt lost the protective effect of cyclic strain on endothelial survival and proliferation [551]. Their study observed a significant reduction in BAD phosphorylation and an increase in cleaved caspase 3 in the Akt deficient group [551]. BAD can lead to expression of other anti-apoptotic proteins [551;552]. Jin et al found that BAD phosphorylation by Akt leads to Bcl-2 expression in vascular endothelium in a rejection model [552]. Akt also directly alters caspase activity. Akt/PKB activation results in phosphorylation of caspase 9 [544-549]. When pro-caspase 9 is phosphorylated it renders it uncleavable for cytochrome C [544-549]. Kang et al observed that Akt/PKB also inhibits caspase 8 [553]. Their data showed that endostatin induced cell death was caspase 8 driven and was mediated by Akt/PKB inhibition [553]. Ohashi et al showed that Akt/PKB can also inhibit caspase 3 activation in microvascular endothelial cell [558]. Akt/PKB also reduces the ability of transcription factors to activate transcription of apoptotic genes (such as the transcription factor FOX) [544-549;555;559]. Akt/PKB phosphorylated FOX loses its transcriptional ability and prevents the expression of apoptotic proteins [555;559]. Skurk et
al as well as Kim et al found that Akt/PKB regulates FOX in endothelial cells and prevents apoptosis through modifying expression of heat shock protein 70 and caspase 8 [555;559].

Akt/PKC can also regulate NO production [301;302;436;562]. Akt/PKB can directly activate eNOS and through PKC can indirectly activate eNOS [301;302;436;562]. In addition, PKC can activate Akt and lead to eNOS activation [301;302;436;562]. Dimmeler et al demonstrated that Akt/PKB can activate eNOS in endothelial cells [301]. Michell et al found that Akt/PKB can directly phosphorylate eNOS and lead to NO production [562]. Ho et al observed that eNOS can modulate Akt/PKB activity [554]. They demonstrated that hyperglycemia induces vascular endothelial cell ROS production which in turn decreases Akt/PKB phosphorylation and therefore results in apoptosis [554].

1.6 ENDOTHELIUM AND ENDOTHELIAL DYSFUNCTION

The endothelium plays a crucial role in maintaining vascular homeostasis. The normal endothelium regulates vascular tone, prevents coagulation and inhibits immune activation [229;236]. Endothelial dysfunction is characterized by impaired NO homeostasis, increased vasoactive peptide release, heightened coagulation properties, and increased immune interaction. Endothelial dysfunction is involved in the pathogenesis of several diseases such as hypertension, atherosclerosis and CAV. [563-575] Furchgott et al demonstrated that the intact endothelium is required for proper homeostasis and response to endothelial cell dependent vasodilatory stimulus [236]. They showed that Ach results in vasoconstriction of aortic rings instead of vasodilation when the endothelium was denuded [236]. Therefore, endothelial injury and dysfunction may lead to heightened and paradoxical vasocontraction when exposed to endothelial-dependent vasodilators [236]. Endothelial dysfunction is a well known cause of atherosclerosis [569-585]. Hollenberg et al and Marti
et al showed that endothelial dysfunction predicts the development of allograft vasculopathy and that the severity of dysfunction is correlated with the degree of intimal thickening [566-568].

Local vascular control depends upon a balance between endothelium-derived vasodilators and constrictors. Endothelial dysfunction is a systemic process that develops in response to chronic inflammation, I/R and other risk factors. It is manifested by a diminished production/availability of NO with coexisting alterations in endothelium-derived contracting factors such as ET-1 [504;586;587]. Such an imbalance predisposes the vasculature to increased tone, altered geometry/remodeling, thrombosis, inflammation, oxidation and proliferation. Evidence suggests that impaired NO release precedes the development of atherosclerosis and serves to reinforce vascular pathology once established. NO is the most important vasodilator and maintains basal vascular smooth muscle tone and resistance to atherosclerosis. In addition to being the main determinant of tone, NO opposes the actions of potent endothelium-derived constrictors such as ET-1, angiotensin and ROS. ROS can also lead to endothelial dysfunction by causing direct vessel injury, increasing the production of ET-1 and other inflammatory markers and also by impairing NO bioavailability [588-591].

There are several causes of endothelial dysfunction such as hypercholesterolemia, oxidative stress, hypertension, I/R, infection (CMV, and Chlamydia) and ET-1 [86;568;586;592-600]. The common pathway by which these factors lead to dysfunction is impairment in NO production. Impaired NO production is associated with increased vascular tone, increased platelet aggregation, increased inflammatory reactions (increased cytokine release and endothelial cell leukocyte adhesion), decreased ROS scavenging
(increased ROS damage and further impairment in NO formation), and altered vasodilator to vasoconstrictor balance. Although ET-1 has been shown to be associated with endothelial dysfunction, atherosclerosis and CAV the mechanisms behind ET-1 induced changes are unknown [15;601;602]. Barton et al demonstrated that ET-1 blockade improves endothelial function in atherosclerotic mice [601]. Bohm et al found that human forearm blood flow in atherosclerotic patients can be improved by ET-1 antagonism [602]. Several investigators have demonstrated that ET-1 adversely affects endothelial function as well as outcomes following cardiac surgery [17;563;603-605]. We have previously shown that ET-1 results in endothelial dysfunction following cardiac transplantation and antagonism with BOS abrogated this effect [17]. Previous investigators have demonstrated that ET-1 modulates eNOS expression as well as NO production [606;607]. Elevated ET-1 levels have also been implicated in the development of transplant coronary disease [33;564;603;608]. It is generally accepted that low levels of ET-1 increase NO production; however, recent evidence suggests that elevated levels of ET-1 (as seen following heart failure and I/R) may impair NO production [607;609]. However, the mechanisms by which elevated levels of ET-1 reduce NO production in endothelial cells have yet to be determined. Oxidative stress can increase ET-1 levels and ET-1 has been demonstrated to increase ROS production in the rat aorta; therefore, alterations in one can led to a feed-forward cycle ultimately resulting in endothelial dysfunction [590;591;610]. In addition, the mechanistic effects of elevated ET-1 levels on endothelial cell ROS generation remain unknown.
1.7 REACTIVE OXYGEN SPECIES

ROS is a family of oxygen radicals ($O_2^-$) and free radicals. Oxygen radicals are also free radical. Other free radicals include NO radical (NO), PNT and hydrogen peroxide. A free radical is a molecule that has an unpaired electron. Important vascular radicals are $O_2^-$ and NO$^\cdot$. ROS are produced in situations of oxidative stress such as I/R or stimulated by cytokines or other vasoactive peptides [142;610-625]. Recent studies have indicated that ET-1 may induce an oxidative burst [610]. ROS production can result in serious cellular damage [620-622;625-640].

ROS are formed by several possible pathways. They include:

1. eNOS/iNOS.
2. NAPDH and NADH oxidase.
3. Mitochondrial pathways (Complex I and III)
4. Xanthine oxidase.
5. Iron.

The first three pathways play significant roles in endothelial cell induced ROS production. eNOS as previously described can produce NO$^\cdot$ when in the uncoupled state. NO$^\cdot$ reacts with $O_2^-$ to form an even more potent radical PNT. eNOS uncoupling occurs when BH$_4$ levels are reduced, L-arginine levels are low or by inflammatory cytokines. I/R, ROS and ET-1 have also been implicated in eNOS uncoupling. iNOS, an inducible form of NOS, produces oxygen radicals when induced and activated. Activation of iNOS occurs following I/R and cytokine stimulation. iNOS exclusively and efficiently produces oxygen radicals. The effects of eNOS/iNOS ROS production are significant. A unique feature is that PNT can oxidize methionine, hydroxylate and nitrate aromatic rings leading to protein and
DNA breakdown and the accumulation of cytotoxic compounds all leading to cellular injury and apoptosis [142-144;146;641]. Clinical effects range from myocardial and endothelial damage to the development of atherosclerosis and CAV [142-144;146;641]. Ma et al and Lopez et al demonstrated that PNT causes myocardial injury and enhances ischemia reperfusion injury to the rat heart [143;144].

NAPDH oxidase is a membrane bound enzyme that also produces oxygen radicals [431;432;610;617;642-650]. Endothelial NADPH oxidase is composed of several subunits. It is composed of cytosolic subunits, a regulatory G-protein and a membrane-bound cytochrome b558 reductase domain [617;651-654]. NADPH can be activated by cytokine stimulation or following I/R [431;432;610;617;642-644;644;645;645-649;649;650;655-659]. ET-1 can also result in NADPH oxidase activation in the rat aorta [610]. NADPH was found to be a player in endothelial dysfunction and atherosclerosis [644;645;655]. Barry-Lane et al demonstrated that NADPH knockout mice display decreased ROS production when stimulated [655]. When these mice were bred with apoprotein-E (apoE) knockout the double knockout mice displayed a significant reduction in atherosclerotic lesions [655]. Therefore, Barry-Lane et al showed that NADPH is involved in the pathogenesis of endothelial dysfunction and atherosclerosis [655]. Wang et al demonstrated the importance of NAPDH oxidase in ischemia reperfusion injury [656]. They found that apocynin, an NADPH oxidase inhibitor, reduces cerebral ischemia reperfusion injury in a gerbil model of carotid artery occlusion [656]. Hoffmeyer et al demonstrated using NADPH oxidase knockout mice that were submitted to myocardial I/R, that the area of myocardial infarction and dysfunction was similar to their control group [660]. Their data indicates that NADPH may not play a role in myocardial injury following I/R [660].
The mitochondria can also be a source of ROS [661-673]. Complex I and III have been demonstrated to generate free radicals [661-674]. Mitochondria can be induced to produce oxygen radicals when ADP is low, Ca\(^{2+}\) level are high or when cytochrome c oxidase is dephosphorylated [661-663;665;669-673]. These three conditions occur during I/R. Therefore, I/R is a potent stimulator of mitochondrial ROS production. The significant impact of mitochondrial ROS generation goes beyond the deleterious effects of the free radical because in a ROS producing state there is a decrease in the formation of ATP which worsens ischemic injury and further increases ROS production. Another potential source of mitochondrial ROS is mitochondrial NOS (mtNOS) [667;675-677]. Boveris et al and Valdez et al demonstrated that the heart does have mtNOS [675;676]. mtNOS under normal conditions is involved in oxygen uptake and NO formation however, when ADP concentrations are low mtNOS becomes an hydrogen peroxide producer [667].

Xanthine oxidase can also produce ROS in endothelial cells however its significance remains debated [622-625]. Xanthine oxidase possesses an interesting property in that it can be released into the circulation from damaged cells and bind to the endothelium where it can produce ROS and result in endothelial dysfunction [622-625]. Following I/R it has been demonstrated that circulating xanthine oxidase levels are elevated [623].

Unbound iron has the ability to catalyze the formation of hydroxyl radicals and enhance the formation of lipid peroxidation [674;678-681]. Iron is usually associated with ferritin or heme. In situations of increased oxidative stress transport and storage proteins are broken down and iron is released leading to accelerated hydroxyl and lipid peroxidation [674;678-681]. Other mechanisms which can generate ROS include myeloperoxidase and lipoxygenase free radical formation.
The deleterious effects of ROS include protein degradation, DNA damage [629;631;682-684], Ca\textsuperscript{2+} accumulation, altered ion and channel function, lipid peroxidation and decreased energy stores (NAD\textsuperscript{+} and ATP) [620-622;625-640]. ROS formation can also lead to apoptosis [626-629]. It has been argued that this effect is beneficial or antioxidant like since removing the ROS generating cell will reduce the oxidative burden. However, if the numbers of affected cells are extensive severe tissue damage can occur. ROS has also been implicated in the development of atherosclerosis and CAV [588;622;685-689]. ROS can initiate atherosclerosis by several mechanisms which include direct endothelial damage leading to activation and inflammatory reaction. ROS can result in endothelial dysfunction, a known pathogenic cause of atherosclerosis. ROS increases LDL peroxidation by increasing macrophage uptake and foam cell formation [686-689]. In cardiac transplantation, ROS can result in primary allograft failure by impairing both myocardial and endothelial function. CAV can also be initiated and enhanced by ROS induced endothelial damage.

The endothelium does posses defense mechanisms against ROS damage. The three defense mechanisms are superoxide dismutase (SOD) (intracellular or extracellular), catalase and glutathione peroxidase [690-696;696-698;698]. The endothelial cell has both intracellular and extracellular SOD [690-698]. Extracellular SOD likely functions to limit I/R injury [691;693-695]. Sjoquist et al demonstrated in a isolated rat heart model of coronary ligation that the addition of extracellular SOD reduced myocardial damage [693]. Abrahamsson et al found that extracellular SOD protected against endothelial dysfunction from ROS [691]. Carlsson et al showed a loss in the ability to handle oxidative stress in mice lacking extracellular SOD [695]. SOD is an enzyme that catalyses the formation of one
hydrogen peroxide and one oxygen molecule from two oxygen radicals and two protons. SOD therefore removes two oxygen radicals per reaction however, accumulation of large amounts of hydrogen peroxide (also a ROS) is deleterious to the endothelial cell. Catalase and glutathione peroxidase are enzymes that catalyse the breakdown of hydrogen peroxide. Catalase converts two hydrogen peroxides into two molecules of water and one of oxygen. Glutathione peroxidase protects the endothelial cell by generating oxidized glutathione from reduced glutathione and hydrogen peroxide. Reduced glutathione is regenerated by glutathione reductase by reducing oxidized glutathione in the presence of NADPH.

1.8 IMMUNOSUPPRESSION

In the early 1950’s organ rejection was quickly realized as the main obstacle to organ transplantation. In the late 1950’s and early 1960’s several strategies were used to improve transplantation survival by modulating the host immune response. The first attempts with total body irradiation had little success. The next step was the use of alkylating agents and anti-metabolites. They offered some benefit against rejection however, their effectiveness was limited especially at the concentrations tolerated by patients. The introduction of CyA in 1978 greatly revolutionized clinical organ transplantation. For the first time, transplant physicians were able to reduce acute rejection and significantly improve survival with acceptable side-effects. Over the next thirty years significant advances were made in the development of immunosuppressive drugs that allowed transplant physicians to more specifically modulate the immune response depending on the patient and organ transplanted. However, it has become apparent that these agents are associated with many side effects including the development of allograft vasculopathy which can limit graft and patient survival.
Immunosuppressive agents can be classified by their class or mechanism of action. There are several mechanisms by which immunosuppressants can reduce rejection. An immunosuppressive agent can have more than one mechanism of immune suppression. The mechanisms of immune suppression are:

1. Inhibition of T-cell activation.
2. Inhibition of T-cell proliferation.
3. T-cell depletion.
4. Inhibition of B-cell proliferation.
5. B-cell and antibody depletion.
6. Inhibition of smooth muscle proliferation (chronic vascular rejection leading to allograft vasculopathy).

Following transplantation there are three phases of immunosuppression. The first is a high dose acute therapy to limit the development of acute rejection. The second is referred to as maintenance therapy which is required to avoid rejection since graft tolerance strategies are not yet available. The third is treatment of acute rejection episodes while on maintenance therapy. These treatment modalities usually require increasing the current regimen dose or adding another immunosuppressant.

Maintenance therapy is crucial for graft and patient survival. Several different classes of immunosuppressive agents can be used for maintenance therapy. The classifications of immunosuppressive drugs are:

1. Steroids (Corticosteroids).
2. Alkylating agents (Cyclophosphamide).
3. Anti-metabolites (Azathioprine, mycophenolate mofetil, and methotrexate).
4. Calcineurin inhibitors (CyA and tacrolimus).

5. Mammalian target of rapamycin (mTOR) inhibitors (SRL and Everolimus).

Corticosteroids, such as prednisone, have both immunosuppressive and anti-inflammatory properties. The immunosuppressive actions of corticosteroids are achieved by reducing inflammatory mediators, decreasing T-cell activation/proliferation and B-cell proliferation. Corticosteroids passively enter the cell and bind to its cytoplasmic receptor. Once the corticosteroid-receptor complex is formed translocation into the nucleus occurs. In the nucleus the complex binds to the glucocorticoid response element and inhibits DNA transcription. In the T-cell, corticosteroids inhibit nuclear factor of activated T-cell (NFAT) which results in decreased production of IL-1, IL-3 and IL-6 as well as INF-γ and TNF-α [699]. The resultant effect of decreased cytokine production is prevention of T-cell activation and proliferation. Corticosteroids also prevent B-cell and macrophage activation.

Alkylating agents such as cyclophosphamide interfere with DNA replication. DNA replication is impaired as a result of alkylation and crosslinking of DNA strands. This form of therapy interferes with actively dividing cells and prevents T-cell and B-cell proliferation. These agents have no effect on limiting T-cell activation.

Anti-metabolites are divided into two types: those that interfere with purine synthesis (azathioprine and mycophenolate mofetil) and those that interfere with pyrimidine synthesis (methotrexate). These agents interfere with DNA synthesis and prevent T-cell and B-cell proliferation. These agents also have no effect on limiting T-cell activation.

Calcineurin inhibitors interfere with T-cell activation by inhibiting calcineurin dependent gene transcription and ultimately prevent IL-2 production. mTOR inhibitors
inhibits TOR induced T-cell and B-cell activation. Calcineurin and mTOR inhibitors will be discussed in more detail in the following section.

1.8.1 Calcineurin Inhibitors

The first clinical trials of CyA were performed in 1979 and greatly revolutionized the transplant world [700-703]. For the first time efficient immunosuppression was achieved with minimal toxicity [700-703]. Discovered in the early 1970’s, CyA is an antifungal product that was derived from *Tolypocladium inflatum Gams*. CyA is a hydrophobic cyclic 11 amino acid peptide.

T-cell activation is a coordinated series of steps. First the T-cell interacts with an antigen through its T-cell receptor. Receptor ligand interaction results in intracellular Ca\(^{2+}\) accumulation which binds calmodulin and forms an activated binding complex with calcineurin. Calcineurin dephosphorylates NFAT causing a conformational change that exposes NFAT’s nuclear localization sequence which results in binding to importin and nuclear translocation. Nuclear NFAT results in transcription of several genes such as IL-2, IL-3, IL-4, INF-\(\gamma\) and TNF-\(\alpha\) leading to T-cell activation and proliferation [699].

CyA’s mechanism of immunosuppression involves inhibition of calcineurin. Calcineurin is a Ca\(^{2+}\) calmodulin-dependent serine/threonine phosphatase. Calcineurin is composed of two subunits: a regulatory calcineurin B and a catalytic calcineurin A [704-708]. The N-terminus of calcineurin A is the catalytic site whereas the C-terminus is the binding site for calmodulin and calcineurin B and also contains the autoinhibitory site. Calcineurin activation requires the binding of Ca\(^{2+}\) to calcineurin B as well as to calmodulin. Calcineurin B is composed of four globular Ca\(^{2+}\) binding domains. The binding of Ca\(^{2+}\) to calcineurin B is required for full activation of the catalytic site.
CyA does not directly inhibit calcineurin. CyA binds to an immunophilin and forms a CyA immunophilin complex. This immune complex binds and inactivates calcineurin leading to decreased NFAT induced transcription and hence decreased T-cell activation and proliferation [709]. Immunophilins are a family of five peptiy-l-prolyl cis/trans isomerases that are divided into two families: The cyclophilins (which bind CyA) and the FK-binding proteins (FKBP) (which bind Tacrolimus). These isomerases are important in protein folding [710].

Tacrolimus, another calcineurin inhibitor, is a common immunosuppressive agent used today. It is also derived from a fungal product of *Streptomyces tsukubaensis*. Similar to CyA, tacrolimus requires binding to an immunophilin to inhibit calcineurin. Tacrolimus binds to FKBP-12 and then complexes with calcineurin which inhibits calcineurin activation.

Shortly after the clinical introduction of CyA it was discovered that it has several deleterious side-effects. These side-effects include nephrotoxicity, hypertension, hepatotoxicity, neurotoxicity as well as cardiotoxicity. CyA has also been demonstrated to have adverse effects on endothelial cells and the development of CAV.

**1.8.2 CyA, Endothelial Dysfunction And Cardiac Allograft Vasculopathy**

Several investigators have demonstrated that CyA exposure leads to endothelial dysfunction [594;711-721]. The mechanisms behind CyA-induced endothelial dysfunction have not been fully elucidated. Three important pathways have been implicated. 1) Altered NO homeostasis [719;722-724]. 2) Increased vasoactive peptide release [725-727]. 3) Increased ROS production [723;728-730].
Morris et al demonstrated that CyA treatment resulted in endothelial dysfunction in renal transplant patients [717]. They showed that forearm blood flow was impaired in CyA exposed patients compared to control [717]. Ovuworie et al observed in renal transplant recipients using brachial ultrasound that endothelial dependent vasodilation was impaired in CyA treated patient versus controls [718]. They found no differences in endothelial independent blood flow between groups [718]. Diederich et al in a rodent model found that CyA results in endothelial dysfunction as well as minor impairment in endothelial independent vasorelaxation [723]. Schrama et al demonstrated endothelial dysfunction due to CyA in transplant recipients [594]. They showed that CyA results in von Willebrand factor and P-selectin upregulation which alters normal anti-thrombogenic properties of the endothelium [594]. Sudhir et al in a canine model of acute CyA exposure found a significant impairment in endothelial dependent coronary artery blood flow [719]. Their study demonstrates the ability of CyA to impair endothelial function acutely indicating that CyA may have direct effects on endothelial function in addition to chronic effects seen after prolonged CyA therapy [719]. Their data also suggested that NO impairment may be a key player in CyA induced endothelial dysfunction [719]. El Hamamsy confirmed Sudhir’s results in a porcine model [714]. They also observed the protective effects of BH4 on preventing endothelial dysfunction [714]. However, they did not analyze whether BH4 protection was due to increased NO production or decreased ROS formation or both [714]. Calo et al revealed that CyA leads to endothelial dysfunction as a result of increased eNOS driven ROS [713], while Oriji et al showed that CyA treatment decreases endothelial NO production [731]. Kou et al’s study supports the hypothesis that eNOS is involved in CyA induced dysfunction [732]. They found that eNOS phosphorylation was reduced in the
presence of CyA [732]. Decreased eNOS phosphorylation resulted in decreased NO production. Enhanced production of ROS species as a cause of endothelial dysfunction was also supported by the studies of Diederich et al and Galle et al [730;733]. Galle et al demonstrated that CyA exposure results in superoxide radical formation and that oxidized LDL heightened the production of superoxides [733]. CyA is known to increase LDL in transplant patients; therefore, CyA increases ROS production while raising LDL levels leading to greater formation of oxidized LDL which in turn causes more endothelial dysfunction and higher ROS formation. CyA induced endothelial dysfunction can also be as a result of increased vasoactive peptide formation [725;734-745]. Abraham et al found that CyA induced endothelial dysfunction was limited by saralasin demonstrating the role of angiotensin in CyA induced vascular [725]. Bunchman et al found that human endothelial cells exposed to CyA produced and released ET-1 [734]. Takeda et al confirmed that ET-1 levels are increased following CyA treatment and that ET-1 antagonism reduces CyA induced hypertension in a rodent model [742-745]. Fogo et al observed improved glomerular filtration rate in CyA exposed rats given BQ-123, an ET-1 antagonist [737]. Petrakopoulou et al found in a clinical study of heart transplant patients that CyA therapy results in microvascular impairment as well as elevated ET-1 levels [746]. Cauduro et al also observed an increased plasma ET-1 level following CyA treatment [747]. Wilasrusmee et al demonstrated that ET-1 leads to endothelial dysfunction and that CyA-mediated capillary dysfunction was abolished by ET-1 antagonism in a capillary tube formation model [748]. Therefore, CyA can induce endothelial dysfunction by impairing NO production, increasing ET-1 and by increasing ROS production through eNOS and ET-1 mechanisms. CyA induced lipidemia adds fuel to the fire. CyA also has been associated with the development
of CAV [594;717;728;749-753]. CyA can influence transplant vasculopathy by increasing plasma lipid concentrations, causing hypertension or by direct injury to the endothelium [753-756]. ET-1 is another mechanism by which CyA can cause CAV. Yamaguchi et al, using a rodent heart transplant model, demonstrated that ex vivo blockade of ET-1 reduces CAV [103]. Hollenberg et al demonstrated that endothelial impairment over time predicts the development of CAV in patients [566;567]. Petrakopoulou et al found that CyA induced microvascular impairment was associated with greater mean intimal area compared to tacrolimus [746].

1.8.3 mTOR Inhibitor

Rapamycin (also known as Sirolimus, SRL) is a product of Streptomyces Hygroscopicus. SRL binds to the same family of immunophilins as tacrolimus (FKBP-12) however instead of blocking calcineurin-dependent T-cell activation it inhibits the mTOR. mTOR phosphorylates proteins involved in cell-cycle regulation playing a crucial role in T- and B-cell proliferation.

mTOR is a serine/threonine kinase that is 2549 amino acids long [757-773]. It is part of the phosphatidylinositol kinase family and is involved in cell cycle control, DNA repair, mRNA translation and nutrient sensing in nearly all cell types [757-773]. It is composed of a regulatory domain and a catalytic domain. The catalytic domain is in the C-terminus of the kinase and downstream is located the FKBP-12 domain (also known as the SRL binding domain). mTOR interacts with its regulatory proteins via a tandem repeat motif region called HEAT. Activation of mTOR occurs by growth factor signaling, nutrient status sensing and sensing of energy status. When stimulated by growth factors or by amino acid sensing, mTOR forms one of two complexes: Complex 1 is formed by the binding of mTOR
and the regulatory factor Regulatory Associated Protein of mTOR (RAPTOR). Complex 2 which is formed by the binding of mTOR and the regulatory factor SRL-Insensitive Companion of mTOR (RICTOR). The final step in mTOR activation is binding to mLST8/GβL regulatory protein which stabilizes the complexes. Complex 1 is SRL sensitive and is involved in growth factor and nutrient signaling while Complex 2 is SRL insensitive and is involved with cytoskeletal regulation. Once activated, Complex 1 results in cell growth and cell division.

mTOR is activated by several growth factors and cytokines such as IL-2 and VEGF, by adequate ATP levels (intracellular) and by abundant amino acid levels (extracellular). Activated mTOR then promotes cell growth, division and survival by phosphorylating and activating transcription factors and Akt/PKB.

mTOR inhibition either by cell starvation, low energy state or by SRL can result in either cell cycle arrest (G1-S phase), autophagy or cell death. SRL leads to immunosuppression by binding to FKBP-12 which then binds and inhibits mTOR. mTOR inhibition of T-cells leads to G1-S phase arrest and blocks the T-cell response to cytokines and hence stops T-cell proliferation and activation.

1.8.4 mTOR, Endothelial Function And Cardiac Allograft Vasculopathy

SRL has both an anti-proliferative effect and a protective effect against the development of CAV in a rodent model [750; 774]. Corbin et al have shown that SRL exposure results in vasomotor relaxation of rat aortic rings in a dose-dependent fashion [775]. However, Jeanmart et al demonstrated that SRL results in greater endothelial dysfunction compared to CyA [715]. CyA results in endothelial dysfunction likely as a result of NO and ET-1 dysregulation. Whether SRL results in endothelial dysfunction or
impairs NO-ET-1 homeostasis remains unclear. Naoum et al showed that SRL treatment can result in eNOS upregulation which may have potential beneficial effects post cardiac transplantation in preventing CAV [776], while Chen et al found decreased eNOS expression in endothelial progenitor cell [777]. The effect of SRL on NO and ET-1 regulation remains largely unknown. Goggins et al found that a single dose of SRL can reduce the development of CAV in a rodent heterotopic cardiac transplant model [778]. Since the SRL dose was given preoperatively the mechanism of SRL protection is unknown. Protection can be as a result of limiting I/R injury, improved NO-ET-1 homeostasis, an anti-proliferative effect or a combination thereof. Schmid et al observed that SRL can reduce the development of CAV and Poston et al found that SRL can reverse CAV in a rodent model of vasculopathy [774,779]. Mancini et al demonstrated that SRL treatment can also decrease the progression of established CAV in cardiac transplant patients [780]. Clinically, SRL has been demonstrated to reduce the incidence, progression and severity of CAV [781], yet the mechanisms by which SRL leads to endothelial protection and prevention of CAV remains unknown.

1.9 CELL DEATH

Cell death can occur by several mechanisms. They can be divided into two categories: apoptotic and non-apoptotic cell death. Non-apoptotic cell death encompasses several different pathways such as necrosis, autophagy and mitotic catastrophe.

Mitotic catastrophe as its name suggests is a form of cell death that occurs when errors in mitosis arise that can not be repaired. Mitotic catastrophe is characterized by the formation of multiple micronuclei and nuclear fragmentation. Mitotic catastrophes occur to
prevent aberrant mitosis from leading to malignant transformation. Therefore, mitotic
catastrophes serve as a defense against tumor formation.

1.9.1 Apoptosis

Apoptosis is a form of programmed cell death also known as cell suicide [782].
Apoptosis has an important role in physiology such as in embryology, T- and B-cell
deletion, and tumor regression [782-785]. Apoptosis can also be pathologic, triggered by a
host of events. These triggers include cytokines, cytotoxic T-cells during rejection, loss of
growth factor stimulation, DNA damage, hypoxia and I/R.

The Apoptotic pathway is mediated by three components: 1) Caspases, cysteine
containing aspartate-specific proteases, 2) Bcl-2 proteins, and 3) Apaf protein [782-802].
Caspases are a family of proteases that mediate apoptosis [782-802]. Caspases are produced
in an inactive proform that requires cleavage for activation [782-802]. Caspase can be
activated by two separate pathways [782-802]. The first pathway is the extrinsic pathway
which is activated by receptor ligand binding [803]. The death receptor on the cell surface is
bound by its ligand and triggers the activation of caspase 8 [794;803]. Triggers of the
extrinsic pathways are FAS ligand (FASL) and TNF superfamily which bind to their
receptors FAS and TNF receptor (TNFRc) [803-809]. FASL-FAS and TNF-TNFRc binding
results in receptor trimerization and activation. FASL-FAS trimerization results in the
recruitment and binding of the death domain containing adaptor (FADD) [802]. TNF-
TNFRc recruits TNF-receptor death domain containing adaptor (TRADD) [802]. Once this
complex is formed the death-inducing signaling complex (DISC) can bind and become
activated. DISC binds and cleaves procaspase 8 into active caspase 8 and activates the
apoptosis process. Caspase 8 then cleaves and activates the effector caspases 3, 6, 7 [792-794; 797-799].

The intrinsic pathway can be activated by both extracellular and intracellular stimuli which include the loss of growth factor stimulation, DNA damage, hypoxia and I/R. The common link between these triggers is that they all signal apoptosis through the mitochondria. The principal player in activation of the intrinsic pathway is the release of cytochrome c from the mitochondria [810-812]. Cytochrome c can be released from the mitochondria by several events including a change in mitochondrial membrane potential or permeabilization of the outer mitochondrial membrane, altered energy status, ROS induced membrane damage and other intracellular stresses such as DNA damage. Released cytochrome c binds to Apaf and procaspase 9 to form the apoptosome [810-815]. Procaspsae 9 is then cleaved and released from the apoptosome. Activated caspase 9 cleaves and activates the effector caspase 3, 6, 7. Caspase 3 can feedback and activate procaspase 9 hence creating a feed forward cycle.

The Bcl-2 family of proteins is involved in apoptosis (intrinsic pathway) at its initial step [816]. Bcl-2 and Bcl-xL are anti-apoptotic proteins and prevent cytochrome c from translocating into the cytosol while Bax and Bid are pro-apoptotic and promote cytochrome c cytosolic release [816-824]. The anti-apoptotic effect of Bcl only occurs at the start of the cascade. If cytochrome c is released into the cytosol then apoptosis occurs regardless of Bcl-2 levels [817]. Bcl-2 family member Bid can link the extrinsic pathway to cytochrome release. Caspase 8 through Bid activation can result in cytochrome c release and initiation of apoptosis (intrinsic pathways) [825].
Apoptosis can also occur in a caspase independent manner through the activation of apoptosis-inducing factor or Endonuclease G [826-828]. Activation of apoptosis results in DNA breakdown forming a characteristic DNA laddering pattern on agarose gels, endonuclease activation, chromatin condensation and cell death.

1.9.2 Autophagy

Autophagy is a normal cellular process that removes unwanted or damaged protein that is independent of the ubiquitin system [829-839]. The process involves the removal of dysfunctional proteins or organelles by specialized lysosomes called autophagosomes. Autophagy leads to cell death when the process becomes excessive or prolonged. Autophagy is regulated by two kinases PI3K and mTOR [829;830;833;836-839]. mTOR inhibits the formation and growth of autophagosomes; therefore, mTOR inhibition leads to autophagy [829;830;833;836-839]. Triggers of mTOR inhibition such as SRL and starvation can lead to autophagy. Its role in endothelial cell death following hypoxia and reperfusion as well as the effect of ET-1 remains to be elucidated.

1.9.3 Necrosis

Always caused by pathologic conditions, necrosis is an unregulated cell death mechanism [831;840]. It can be induced by overwhelming inflammation or I/R. It is characterized by enzymatic degradation and protein denaturation. The cellular insults lead to membrane damage, organelle swelling and lysozyme release. Intracellular pH drops resulting in proteolytic enzyme activation and protein breakdown. Mitochondrial disruption results in poor energy status. Unlike the ordered DNA breaks that occurs with apoptosis, necrosis results in DNA clumping and random DNA breaks. Finally, the excessive intracellular degradation and cellular swelling leads to cell rupture and death.
1.10 ENDOTHELIN-1

Discovered in 1988 by Yanagisawa et al, ET-1 is the most potent vasoconstrictor known [841]. ET-1 is part of the endothelin family [842]. Three endothelin peptides have been described: ET-1, ET-2 and ET-3 [842]. ET-1 is produced principally by endothelial cells but SMC have been demonstrated to release ET-1 [841;843;844]. ET-2 is found in the kidney and intestine. ET-3 is produced in the brain [845]. Each ET peptide is encoded on a different gene. The gene encoding for ET-1 is on chromosome 6 [846;847]. The gene encodes for a 202 amino acid preproET [844;848]. The preproET from is cleaved into a 38 amino acid precursor called BigET-1 by endopeptidases. Big ET-1 is secreted by the endothelial cell and converted by the enzyme ECE into the 21 amino acid ET-1 peptide [849-853]. ECE is located on endothelial cells especially in the lung vasculature [849-852]. ET-1 contains two intrachain cysteine disulfide bonds (between positions 1 and 15 and between positions 3 and 11) and a terminal tryptophan (Figure 9). The disulfide bonds and the terminal tryptophan are crucial for normal ET-1 activity. Kimura et al demonstrated that removal of either the disulfide bonds or the terminal tryptophan results in a significant decrease in potency [854].

ET-1 production is regulated by several factors [855-870]. The promoter region of ET-1 contains activator protein-1 (AP-1), GATA-2, CAAT-binding factor (NF-1), NFκB and acute phase regulatory elements. Cytokines (IL-1, TGF-β and TNF-α), growth factors, NO and ET-1 also regulate the production of ET-1 [855-870]. Kurihara et al and Gonzalez et al found that TGF-β can stimulate ET-1 production in vascular endothelial cells [862;871]. Decreased NO production and release reduces formation and release of ET-1 [856;858;861]. Ohkita et al showed in a porcine aortic cell that the NO donor, FK409,
abrogates basal and TNF-α induced ET-1 production [858]. They also showed that NF-κB activation results in ET-1 production and that NO inhibits ET-1 synthesis partially through NF-κB inhibition [858]. Dumont et al also found that NO augmentation with low dose L-arginine reduces ET-1 levels [861]. Chen et al confirmed the same inhibitory action of NO on ET-1 synthesis in cardiomyocytes [856]. Exposure of endothelial cells to hypoxia or I/R can result in ET-1 production. Yamachita et al demonstrated that endothelial cells exposed to hypoxia results in ET-1 production through the transcriptional factor hypoxia-inducible factor-1 (HIF-1) [860]. Their data also supports the importance of AP-1, GATA-2 and NF-1 in ET-1 synthesis since HIF-1 alone is unable to bind to the promoter region [860]. The HIF-1 binding site is inverted and therefore requires the other transcriptional elements for induction of ET-1 transcription [860]. Hu et al also observed that HIF-1 results in ET-1 production in cultures endothelial cells through an upstream promoter [867]. Following I/R several investigators have demonstrated increased ET-1 levels [872-877]. Keltai observed, in a canine model of myocardial infarction, an increased ET-1 gene expression as measured by increased ET-1 mRNA levels and confirmed increased plasma ET-1 levels [877]. Tsuji et al also showed using a canine model that I/R increases ET-1 levels [876]. Brunner et al revealed that the main source of ET-1 from an ischemic heart was the endothelium [873]. They demonstrated that ET-1 released from the coronaries of ischemic rat hearts was reduced significantly when the endothelial cells were removed by collagenases [873].

ET-1 binds to two ET-1 receptors to elicit its effects [878-883]. They are known as ET_A and ET_B receptors [878-882;884]. These receptors are found in virtually every tissue. ET_A and ET_B are rhodopsin-like seven transmembrane spanning domain receptors coupled to G proteins [885;886]. Several members of the G protein family play a role in ET receptor
signaling [887-901]. They include Gs, Gq, and Gi. G\(_{\alpha s}\) activate and G\(_{\alpha i}\) inhibit adenylyl cyclase [887-901]. G\(_{\alpha q}\) activates phospholipase C. Binding of ET-1 to its receptor results in a conformational change and coupling to a G protein. The given G\(_{\alpha\beta\gamma}\) hydrolyzes GTP into GDP and dissociates into G\(_{\alpha}\) and G\(_{\beta\gamma}\). Each dissociated subunit can result in different effector activation or inhibition. Several effector kinases are either activated or inhibited by ET-1 depending of cell type and receptor type [892-894; 898]. These kinases include guanylyl cyclase, adenylyl cyclase, phospholipase C, phospholipase A2, and PI3K. Effector activation or inhibition leads to increased or decreased production of second messengers. These second messengers include cGMP, cAMP, IP\(_3\), DAG, Ca\(^{2+}\), phosphatidic acid, and many others. ET-1, therefore, has the potential to alter PKA, PKB, and PKC activity. Depending on cell and receptor type, ET-1 modulates various signaling pathways (PKA, PKB, and PKC) and results in several physiologic effects.

**1.10.1 Effects Of ET-1**

Since its discovery, ET-1 has been found to have several effects. The first role attributed to ET-1 was vasoconstriction [843; 902; 903]. ET-1 induces smooth muscle vasoconstriction by activating phospholipase C leading to DAG and IP\(_3\) formation resulting in Ca\(^{2+}\) accumulation and smooth muscle contraction [843; 902; 903; 903-906]. The constrictor effect of ET-1 applies to the vascular smooth muscles, tracheal and airway smooth muscle and to myometrial cells [843; 902; 903; 903-906]. However, the degree of contraction varies between SMC types [903]. The myocyte responds in many ways to ET-1 exposure. ET-1 treatment results in myocyte contraction and is a stimulus for hypertrophy. ET-1 also has effects on the kidney, musculoskeletal system and the central nervous system. ET-1 causes renal artery vasoconstriction and can decrease the glomerular filtration rate.
ET-1 is also important in cardiovascular and neural development [907;908]. The endothelium plays an important role in vascular homeostasis. The balance between NO and ET-1 has a crucial role in maintaining this homeostasis. ET-1 produced by the endothelium [909] results in smooth muscle contraction and vessel vasoconstriction. However, ET-1 also affects the endothelium. ET-1 has been demonstrated to regulate NO and eNOS synthesis in the endothelial cell [606;607]. Whether ET-1 increases or impairs NO production from endothelial cells remains controversial. It is generally accepted that low levels of ET-1 increase NO production; however, recent evidence suggests that elevated levels of ET-1 may impair NO production [607;609]. Dong et al and Sugiyama et al found that ET-1 increases ROS production from endothelial cells [607;910]. ROS can also increase ET-1 production therefore, elevated ET-1 levels can heighten its own production by increasing ROS [590;591;610]. The resultant cycle can significantly injure the endothelium and further impair NO and ET-1 homeostasis. In addition, ET-1 induced NO impairment can maintain or further increase ET-1 levels as NO is known to inhibit ET-1 production [240;858;911;912]. ET-1 also activates the endothelium allowing for leukocyte adhesion and activation [913;914].

1.10.2 Effects Of ET-1 On Ischemia/Reperfusion

ET-1 is produced and released by endothelial cells following ischemia [875]. ET-1 results in several deleterious effects during I/R [19;915-923]. We have previously found that ET-1 antagonism with BOS improves both myocardial and endothelial function following cardiac transplantation [17]. We demonstrated that ET-1 plays a role in myocardial and coronary vasculature dysfunction [17]. Gupta et al, using a rodent model of myocardial I/R, observed the protective effects of ET-1 antagonism with BOS [918]. Their data showed that
ET-1 results in injury likely as a result of increased oxidative stress and decreased antioxidant protection (lower catalase, SOD and reduced glutathione) since ET-1 antagonism abrogated these effects [918]. Following I/R Fernande et al demonstrated that the coronary vasculature is more sensitive to ET-1 and that this increased sensitivity is due to altered NO homeostasis [915]. Therefore, the effects of ET-1 may be enhanced following I/R since the vasculature becomes more sensitized to ET-1. Hiramastu et al in a neonatal lamb model of hypothermic cardioplegic arrest demonstrated that ET-1 perfusion worsens myocardial injury and that treatment with BE-18257B, an ET<sub>A</sub> antagonist, reduced myocardial injury [919]. Their study supports the previous observations that ET-1 is involved in ischemic injury [919]. The protective effects of ET-1 antagonism were also demonstrated in a porcine liver transplant model [916]. Fukunage et al showed that TAK-044 (ET-1 antagonist) improved survival as well as reduced endothelial cell injury [916]. Szabo et al showed that selective ET<sub>A</sub> or ET<sub>B</sub> receptor antagonism reduces both myocardial and endothelial injury preventing dysfunction following cardiac transplantation [924].

1.10.3 Effects Of ET-1 On Atherosclerosis and CAV

Elevated ET-1 levels have also been implicate in the development of atherosclerosis and CAV [33;564;586;601;603;605;608;748;925-928]. ET-1 through impairment in NO homeostasis, endothelial cell activation and production of ROS results in endothelial dysfunction and may induce the development of atherosclerosis and CAV. Barton et al demonstrated using ApoE knockout mice that ET-1 blockade restores both endothelial dysfunction and abrogates atherosclerosis [60]. Mawatari et al found that the aorta of atherosclerotic rats have higher expression of ET-1 compared to normal [587]. Winkles et al observed in human endothelial cells from atherosclerotic patients a higher
expression of ET-1 mRNA compared to normal [928]. Lerman et al found elevated plasma ET-1 levels in patients with symptomatic coronary artery disease and that the ET-1 levels correlated with severity of disease [929;930]. They also demonstrated that ET-1 immunoreactivity was associated with endothelial dysfunction and early atherosclerosis [929;930]. Ravalli et al showed an increased ET-1 immunoreactivity in human coronary arteries with CAV [33]. Berkenboom et al demonstrated that early following cardiac transplantation there is a significant correlation between plasma ET-1 levels and degree of coronary dysfunction [603]. Ferri et al showed that myocardial interstitial ET-1 expression was associated with the development of CAV [32]. Wexberg et al observed in cardiac transplant patients that those with BigET-1 levels greater than 2fmol/L had reduced coronary flow reserve as well as increased coronary intimal hyperplasia between 4-5 years after transplant [931]. Weis et al found that coronary vascular function was impaired at one year in cardiac transplant patients compared to nontransplant patients [605]. They also showed that myocardial ET-1 mRNA expression was increased in those transplanted patients versus nontransplanted patients [605]. Yamaguchi et al revealed that ET-1 blockade, with antisense oligodeoxynucleotides of ET-1, prior to heterotopic heart transplantation reduced the development of CAV [103]. They demonstrated that ET-1 is involved in the initiation of CAV [103]. Therefore, ET-1 plays a crucial role in both atherosclerosis and CAV.

1.10.4 Bosentan

BOS is a dual ET\textsubscript{A} and ET\textsubscript{B} antagonist. Derived from the less potent Ro 46-2005 [Ro 46-2005 (4-tert-butyl-N-[6-(2-hydroxyethoxy)-5-(3-methoxy-phenoxy)-4-pyrimidinyl]-benzenesulfonamide]. BOS (formally Ro 47-0203, 4-tert-butyl-N-[6-(2-hydroxy-ethoxy)-5-
(2-methoxy-phenoxy)-2,2'-bipyrimidin-4-yl]-benzenesulfonamide) is a competitive antagonist of the ET\textsubscript{A} and ET\textsubscript{B} receptor. It is principally used clinically to treat pulmonary hypertension. Experimentally, BOS has been demonstrated to reduce endothelial dysfunction in various settings such as atherosclerosis and transplantation [17;918;932-934]. We have previously demonstrated the beneficial effects of BOS in preserving endothelial function in diabetes, following transplantation and following elevated levels of C-reactive protein [17;933;934].
2.1 HYPOTHESIS

Endothelial dysfunction is the hallmark of several pathological states such as atherosclerosis, CAV and primary cardiac allograft dysfunction [563-568;581-585;935]. Endothelial dysfunction occurs in response to chronic inflammation, I/R and other risk factors. It is characterized by a decreased production/availability of NO with coexisting increased production of ET-1 [504;586;587]. ET-1 has been associated with endothelial dysfunction, ischemia reperfusion injury, allograft failure and CAV [17;33;563;564;603-605;608]. The mechanism behind such dysfunction remains unclear. Furthermore, the effect of ET-1 on human endothelial cell NO production remains controversial.

We have previously demonstrated that BOS limits the development of primary allograft failure and preserves endothelial function in a porcine model of cardiac transplantation [17]. This study suggested the possible beneficial role of ET-1 antagonism in preserving endothelial function [17]. We have also observed using this same model that hypoxia and reperfusion (H/R) results in allograft injury however to a lesser extent than I/R [17;100;936]. The mechanisms behind H/R induced human endothelial cell injury as well as the protective effects of BOS remains to be studied.

ET-1 has been speculated to play a role in immunosuppression induced vascular dysfunction. CyA has been associated with the development of hypertension and CAV. Previous studies suggested that NO – ET-1 homeostasis is involved in CyA induced dysfunction [719;723;726;734;744;937-942]. The mechanisms behind CyA induced vasomotor dysfunction requires further investigations. SRL is associated with reduced development of CAV however, whether SRL preserves endothelial function is unknown.
Protein kinases such as PKA, PKB and PKC play important roles in several signaling pathways. They have been implicated in NO homeostasis, ROS production, ischemia reperfusion injury and cell survival. The precise role of these kinases on the human endothelial response to elevated levels of ET-1 and to H/R remains to be elucidated.

ET-1, H/R and CyA are associated with limiting the outcomes following cardiac transplantation. Therefore, limiting ET-1, H/R and CyA induced endothelial injury will likely reduce the development of graft failure and CAV. Understanding the mechanisms behind such dysfunction will lead to the development of pharmacologic strategies to improve outcomes following transplantation. We therefore, propose three investigations designed to:

1) Evaluate the effects of elevated levels of ET-1 on human endothelial cell NO homeostasis and ROS generation as well as the role of protein kinases (PKA, PKB and PKC).

2) Evaluate the effects of H/R on human endothelial cell function and survival as well as the role of ET-1 and protein kinases (PKA, PKB and PKC).

3) Evaluate the effects of CyA and SRL on vasomotor function as well as NO – ET-1 homeostasis.

4) Determine the potential benefit of BOS in the above three studies.

5) Determine the beneficial effects on BH4 in limiting CyA induced dysfunction.

To complete these studies, we employed an endothelial cell culture model in addition to a rodent model to assess the in vivo correlates of our in vitro observations.
We hypothesized the following:

1. ET-1 impairs NO homeostasis and increases ROS generation through PKA, PKB and PKC dependent pathways.

2. Hypoxia and reperfusion alters PKA, PKB and PKC activation leading to ROS generation, impaired NO production and increased apoptosis.

3. ET-1 exacerbates hypoxia and reperfusion induced effects.

4. CyA impairs vasomotor function while SRL limits vasomotor impairment.

5. BOS therapy limits ET-1, hypoxia, reperfusion and CyA induced endothelial injury.

Our studies aim to answer several questions:

Cell Culture Experiments:

1) Elevated levels of ET-1 impair human endothelial cell NO production

2) ET-1 induced NO impairment is mediated by alterations in protein kinases (PKA, PKB, and PKC).

3) NO impairment is mediated by a change in eNOS expression, activity and localization.

4) ET-1 exposure will alter caveolin-1 and iNOS expression.

5) ET-1 increases endothelial cell ROS production.

6) ROS production is mediated by eNOS, iNOS and NADPH oxidase activation.

7) ET-1 mediates ROS production by alterations in PKA, PKC and PKB activity.

8) BOS can prevent ET-1 induced injury.

9) ET-1 plays a role in H/R induced endothelial dysfunction and cell survival.

10) Elevated ET-1 levels will exacerbate H/R injury.
11) Endothelial cell death is mediated through apoptosis, necrosis and autophagy.

12) H/R activates both the intrinsic and extrinsic apoptotic pathways.

13) H/R and ET-1 mediate their effects by modulating protein kinase activity (PKC, PKB and PKA).

14) H/R results in NO impairment and ROS production by altering eNOS expression and localization.

15) H/R will activate NADPH oxidase and induce iNOS expression.

16) We also propose that BOS can prevent ET-1 and H/R induced injury and restore normal vascular homeostasis. BOS therapy during hypoxia will prevent reperfusion injury.

17) Isoform specific PKC modulation alters cell survival as well as NO and ROS formation.

Rodent Model Of Vasomotor Dysfunction:

18) CyA causes vasomotor impairment.

19) CyA alters NO – ET-1 homeostasis.

20) CyA leads to ROS production and oxidative injury.

21) SRL preserves vasomotor function.

22) ET-1 antagonism and NO augmentation will prevent CyA induced vasomotor dysfunction.

The results of these studies will provide new information about endothelial dysfunction induced by ET-1, H/R and CyA. In addition, these investigations will provide the mechanisms behind such dysfunction and suggest potential treatment strategies. Furthermore, our data will demonstrate the potential role of ET-1 antagonism and NO
augmentation in endothelial protection. Finally, results of our in vitro and in vivo studies would form the basis for clinical studies with the ultimate goal to reduce endothelial dysfunction in the cardiac patient. The ability to reduce endothelial dysfunction may limit the burden of atherosclerosis and heart failure as well as improve the outcomes following cardiac transplantation. Since cardiovascular disease account for the greatest morbidity and mortality in the Western World the impact of such benefit would be significant.
CHAPTER THREE

THE ROLE OF PROTEIN KINASES IN ENDOTHELIN-1 INDUCED ENDOTHELIAL DYSFUNCTION:
ALTERED NITRIC OXIDE AND FREE RADICAL REGULATION
3.1 INTRODUCTION:

The endothelium plays a key role in vascular homeostasis through the release of autocrine and paracrine substances. In addition to vasodilation, a healthy endothelium is anti-atherogenic because of its inhibition of platelet aggregation, SMC proliferation and leukocyte adhesion [229;236]. Endothelial dysfunction is the initiator of several pathological states such as atherosclerosis, transplant coronary artery disease and primary cardiac allograft dysfunction [563-568;581-585;935].

Vascular homeostasis depends upon a balance between endothelium-derived vasodilators (such as NO) and constrictors (such as ET-1). Endothelial dysfunction is manifested by a diminished production/availability of NO with coexisting alterations in endothelium-derived contracting factors such as ET-1 [504;586;587]. Endothelial dysfunction can also be precipitated by ROS leading to increased ET-1 production and impaired NO bioavailability [588-591].

Several investigators have demonstrated that ET-1 adversely affects endothelial function [17;563;603-605] as well as modulates NO regulation [606;607]. The ultimate effect of ET-1 on NO production remains unclear and the mechanisms by which elevated levels of ET-1 reduce NO production in endothelial cells have yet to be determined. In addition, the mechanistic effects of elevated ET-1 levels on endothelial cell ROS generation remain unknown.

PKC plays an important role in several signaling pathways. Several investigators have demonstrated that ET-1 alters PKC translocation and activity [163;943] as well as modulate PKA activity is several cell types (smooth muscle, cardiomyocyte and neutrophils) [944-946]. Recent evidence suggests that PKA activation may lead to NO production [504].
The role of PKA in the response of endothelial cells to ET-1 has not been extensively studied. Akt/PKB has been demonstrated to regulate eNOS activity [300;301]. Akt kinase phosphorylates and activates eNOS [301;302]. However, little is known whether ET-1 impairs NO bioavailability through alterations in Akt/PKB.

Our investigations assess the effect of ET-1 on NO and ROS production in human endothelial cells. We also explored the role of protein kinases in ET-1 induced endothelial dysfunction. In that aim we used an endothelial cell culture model to assess the following hypothesis.

We hypothesized the following:
1) Elevated levels of ET-1 impair endothelial cell NO production via a PKC-mediated change in eNOS expression, activity and localization.
2) ET-1 increases endothelial cell ROS production.
3) ET-1 alters both PKA and PKB activity leading to endothelial dysfunction, altered NO and ROS production as well as increased membrane bound eNOS over cytosolic eNOS. 4) ET-1 induced ROS production is principally driven by eNOS uncoupling.
5) BOS can prevent ET-1 induced injury and restore normal vascular homeostasis.

3.2 METHODS:

3.2.1 Endothelial Cell Cultures

Cell culture experiments were performed on human saphenous vein endothelial cell (HSVEC) cultured in 10cm diameter dishes at 37°C and 5% CO₂ in 8ml of medium Dulbecco’s modified Eagle’s medium (DMEM) (VEC Technologies, USA) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100mg/ml streptomycin optimized for endothelial cells. Cells passaged 2 to 4 times and aged between 14 to 30 days from the time
of primary culture were used for this study. We examined the effects of our interventions on eNOS/iNOS/caveolin-1 expression, eNOS localization, NO and ROS production, isoform specific PKC translocation, Akt/PKB phosphorylation, PKA and PKC activity. Cells were treated with 100nM of ET-1 (Sigma, Canada), BOS (10µM, Actelion Pharmaceuticals Ltd, Switzerland), ET-1+BOS (n=16 per group) for 30 minutes or 24 hours. Cells treated with inactive vehicle (0.05% dimethylsulfoxide (DMSO)) were used as controls. The dose of ET-1 was chosen to completely bind the ET\textsubscript{A} and ET\textsubscript{B} receptors and to maintain high levels for the 24-hour incubation period [603]. To modulate the effects of PKC, we employed two antagonists with different mechanisms of action; calphostin C (Cal; 200nM) and chelerythrine (Chel; 1µM) (Sigma, Canada). Chel inhibits the ATP binding site whereas Cal interferes with the lipid cofactor-binding site of PKC. To stimulate PKC activity, the PKC agonist Phorbol 12-myristate 13-acetate (PMA; 10 nM) (Sigma, Canada) was employed. The concentrations of these agents have been previously validated and published by our laboratory [183]. To inhibit PKC\textsubscript{\lambda}, cells were treated with 10 µM atypical inhibitor (ZI) for 24 h. The myristoylated PKC inhibitor ZI was synthesized by the Hospital for Sick Children Peptide Synthesis Laboratory (Toronto, ON). The purity of the peptide was >95% as determined by high-pressure liquid chromatography and mass spectroscopy. The peptide sequence from N-terminus to C-terminus is SER-LLE TYR-ARG-ARG-GLY-ALA-ARG-ARG-TRP-ARG-lys-LEU. PKC\textsubscript{\delta} inhibition was achieved with the use of Rottlerin (DI) (5µM) at a concentration that inhibits only PKC\textsubscript{\delta} and not PKC\textsubscript{\epsilon} (Cedarlane Laboratories Limited, Hornby, Ontario). PKC\textsubscript{\epsilon} inhibition was achieved by using a myristoylated PKC\textsubscript{\epsilon} inhibitory peptide (EI) (100nM) with the following sequence GLU-ALA-VAL-SER-LEU-lys-PRO-THR (Santa Cruz Biotechnology Inc, Santa Cruz, CA). To activate PKA 10µM
of Forskolin (FOS) was used while 10µM H89 (N-[2-(p-Bromocinnamylamino)ethyl]-5-
isoquinolinesulfonamide \cdot 2HCl ) was used to inhibit PKA (Sigma, Canada). 20µM SH5
(Kamiya Biomedical Company, Seattle, WA) was used as our Akt/PKB inhibitor. Antioxidants used in our protocols were Sepiapterin (eNOS cofactor, promotes NO
formation while reducing ROS production) (10mM), N-nitro-L-arginine-methyl-ester (L-
NAME; NOS inhibitor) (1mM), Apocynin (4-hydroxy-3-methoxy-acetiphenone; NADPH
oxidase inhibitor) (1mM), Tiron (TI; specific superoxide anion) (100mM) and Tempol (TE;
SOD mimetic) (1mM) (Sigma, Canada).

3.2.2 NO Production

Cell culture supernatants were collected at 30 minutes and 24 hours following
treatment. NO production was detected spectrophotometrically by measuring its final stable
equimolar degradation products, nitrite and nitrate. Total nitrite was quantified after the
reduction of all nitrates with nitrate reductase. After the conversion of nitrate to nitrite, total
nitrite was determined spectrophotometrically at 540 µm by employing the Griess reaction.
(µQuant Universal Microplate Spectrophotometer, Bio-Tek Instruments, Vermont, USA).
Software used for analysis was the KC4 software version 4.0 form Bio-Tek instruments.
Nitrite concentrations were calculated from a standard curve constructed over the linear
range of the assay and expressed as µmol/L per milligram protein. Nitrate concentration was
calculated by the following equation ‘total nitrite-nitrite’. Sample absorbance values were
corrected by subtracting the absorbance value of blank wells. The amount of nitrite
produced was normalized against total cellular protein, assessed using the Bradford method
[947].
3.2.3 Reactive Oxygen Species Formation

The formation of ROS was detected by loading cultured cells with the fluororope dichlorodihydrofluorescein diacetate (H$_2$DCFDA) (Molecular Probes, Eugene, OR). H$_2$DCFDA is converted into a nonfluorescent polar derivative by cellular esterase after incorporation into cells. This compound becomes fluorescent after conversion by ROS into 2’, 7’-dichlorofluorescein (DCF).

Experiments were performed in 96-well plates. Each well was loaded with 10 000 cells which were allowed to grow until confluence. Prior to treatment, cells were washed three times in PBS and then incubated with 100mM of H$_2$DCFDA for 30 minutes at 37°C. After cell labeling with H$_2$DCFDA each well was washed three times with DMEM without serum. After washing, treatment was added to each well (200µl) and incubated for 24 hours at 37°C. Cells were incubated with H$_2$O$_2$ (25µM) as a positive control. At the end of treatment ROS production was measured by fluorescence at an excitation wavelength at 485nM and emission of 535nM using CytoFluor software analysis version 4.2.1. (CytoFluor multi-well plate reader Series 4000, PerSeptive Biosystem) Sample absorbance values were corrected by subtracting the absorbance from blank wells. Comparisons between groups were performed with the following equation (100 * corrected sample fluorescence values / corrected control fluorescence value).

3.2.4 Protein Expression

Western immunoblotting was performed on total cell lysates using chemiluminescence for detection of iNOS, eNOS, and caveolin-1. Following treatment for 30 minutes or 24 hours in 10cm plates, total cell lysates were obtained as follows: Cells were washed in Phosphate buffered saline (PBS) and collected with 1 ml of ice-cold TRIS-
buffered saline (TBS) (10mM Tris, 0.1M NaCl) containing protease inhibitors (leupeptin, aprotinin, and phenylmethysulphonylfluoride (PMSF) at 0.5µl/ml and pepstatin at 0.7µl/ml). Harvested cells were then centrifuged for 10 minutes at 4°C. Following centrifugation, the cells were resuspended in TBS containing 1% Triton, sonicated on ice and vortexed on ice for three cycles of 10 minutes. Total cell lysates were divided into two aliquots (one for protein determination and the other for western blot analysis) and then frozen in liquid nitrogen and stored at -80°C until ready for analysis. The expression of iNOS, eNOS, and caveolin-1 were determined with the use of protein-specific monoclonal antibodies (BD Biosciences, Canada). Samples were separated using 4% stacking and 10% running tris-glycine sodium dodecyl sulfate-polyacrylamide electrophoresis gels. Gels were then transferred to polyvinylidene fluoride membranes (PVDF). Blocking was performed for 1 hour at room temperature. The blots were stained with monoclonal IgG at a dilution of 1:2500 for 12 hours at 4°C, washed and then incubated with secondary antibody for 1 hour at room temperature. Comparisons between groups were performed using densitometric analysis corrected for β-actin expression. No differences in β-actin protein expression were seen between groups at any time point.

### 3.2.5 PKC Translocation/Activity and eNOS Translocation

Determination of eNOS and isoform specific PKC translocation was performed by analysis of cytosolic and membrane fractions of HSVEC in order to calculate a membrane-to-cytosolic (M/C) ratio. Following treatment for 30 minutes or 24 hours in 10cm plates, cells were washed in PBS and collected with 1 ml of ice-cold TBS (10mM Tris, 0.1M NaCl) containing protease inhibitors (leupeptin, aprotinin, and PMSF at 0.5µl/ml and pepstatin at 0.7µl/ml). Harvested cells were then centrifuged for 10 minutes
at 4°C. Following centrifugation, the cells were resuspended in TBS, sonicated on ice and re-centrifuged for 10 minutes as above. The resultant supernatant represented the soluble cytosolic fraction. The pellet was resuspended in cytoskeletal buffer (150mM NaCl, 30mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 1mM PMSF, 5mM EDTA, and 1% Triton X-100) vortexed for three cycles of 10 minutes and centrifuged to yield the membrane fraction in the supernatant. Both fractions were divided into two aliquots (one for protein determination and the other for western blot analysis) and then frozen in liquid nitrogen and stored at -80°C until ready for analysis. Purity of the cytosolic and membrane fractions was determined by confirming the absence of membrane bound Ca\textsuperscript{2+}-ATPase in the cytosolic fraction or β-actin in the membrane fraction (Figure 10). Our preliminary studies indicated that only the α,δ,ε, ι and λ PKC isoforms are present in this cell population. Therefore, each blot was stained with these isoform specific antibodies. Blots were then scanned using a commercially available software program (Quality one, BioRad, California) and each band assessed densitometrically.

3.2.6 PKB phosphorylation

Akt/PKB phosphorylation was determined by using Western immunoblotting. Total cell lysates were used for analysis using the method described above except for the use of 0.05% Triton instead of 1% Triton. The expression of total Akt, serine-473 phosphorylated Akt (ser-pAkt) and threonine-308 phosphorylated Akt (thr-pAkt) were determined with the use of protein-specific monoclonal antibodies (BD Biosciences, Canada). Samples were separated using 4% stacking and 10% running tris-glycine sodium dodecyl sulfate-polyacrylamide electrophoresis gels. Gels were then transferred to polyvinylidene fluoride
membranes. Blocking was performed for 1 hour at room temperature. The blots were stained with polyclonal IgG at a dilution of 1:5000 for 12 hours at 4°C, washed and then incubated with secondary antibody for 1 hour at room temperature. Each sample was used on three separate blots, one for each Akt antibody in order to make direct comparisons of phosphorylated to total Akt. Comparisons between groups were performed by deriving the ratio of phosphoAkt to total Akt. No differences in β-actin protein expression were seen between groups at any time point.

3.2.7 Protein Determination

Protein determination was performed using the Bio-Rad DC protein dye kit, (BioRad, California) a modification of the Bradford method [947]. Samples were diluted 1:10 and placed into a microtube. Standards were also prepared by serial dilution of bovine serum albumin (10mg/ml) to achieve concentrations of 0.05mg/ml, 0.1mg/ml, 0.25mg/ml, 0.5mg/ml, and 0.77mg/ml. Once prepared, 10µl of samples and standards were transferred into another microtube where 50µl of Working Reagent A and 400µl of Working Reagent B were added to each tube. 200µl of each sample was transferred into 96-well plates and incubated for 15 minutes at room temperature. Following incubation, protein concentration was measured spectrophotometrically at an absorbance (Abs) wavelength of 750nm. (μQuant Universal Microplate Spectrophotometer, Bio-Tek Instruments, Vermont, USA). The KC4 software from Bio-Tek instruments was used for analysis. Sample absorbance values were corrected by subtracting the absorbance value of blank wells. Protein concentration was determined by the following equation: Sample Protein Concentration (mg/ml) = (((Sample Abs750 - Blank Abs750)/ m) x dilution factor) were m is the slope of the blanked standard curve passing through zero.
3.2.8 PKC/PKA Activity

Cells were harvested as described above following the treatment of interest. Following centrifugation for five minutes at 50,000 rpm at 4°C, cells were washed in 1 ml of PBS. This step was repeated twice. The third period of centrifugation was performed at 140,000 rpm for 20 minutes at 4°C. The supernatant was then discarded. The remaining pellet was frozen in liquid nitrogen and stored at -80°C. The day of analysis the pellets were sonicated on ice in 100 µl of sample preparation buffer (SPB) containing 0.05% Triton X-100, 1 mM PMSF, 50 mM β-mercaptoethanol, 0.1 mM sodium orthovanadate, and 1 mM sodium fluoride. Following sonication, cell extracts were transferred to 10 ml polycarbonate tubes on ice. Each tube was centrifuged at 320,000 rpm for 60 minutes at 4°C. The supernatant was divided into two portions (one for protein determination and the other for activity determination). The samples were then processed and added to a commercially available PKC assay kit (Calbiochem, San Diego, California) to determine protein-adjusted activity. For both PKC and PKA activity the samples were diluted 1:2 and 12 µl added to pseudosubstrate-coated wells. To each well was added 108 µl of component mixture (component mixture contains Ca^{2+} 20 mM CaCl_2, 20 µM cAMP (for PKA assay), 1 mM ATP and 500 µg/ml of PS (for PKC assay). The samples were then incubated for 20 minutes at room temperature. Stop solution was added followed by five cycles of washing with the use of a wash solution. Biotinylated antibody 2B9 (100 µl) was added and incubated for 60 minutes at room temperature. After incubation, the wells were washed five times and peroxidase-conjugated streptavidin was added for 60 minutes at room temperature. Following another cycle of washing, 100 µl of substrate solution was added to each well for five minutes at room temperature. The reaction was stopped using a stop solution and the...
optical density read at 490nm in a microplate reader (µQuant Universal Microplate Spectrophotometer, Bio-Tek Instruments, Vermont, USA). Software used for analysis was the KC4 software from Bio-Tek instruments. Activity was determined colorimetrically at 490 nm and is displayed as arbitrary units per mg of protein (U/mg).

3.2.9 PKCλ immunoprecipitation and activity

PKCλ was immunoprecipitated from total cell extract. Total cell extracts (300µg) were precleared by incubation with protein G-Sepharose (100 µl/ml lysate) at 4°C for 20 minutes on a rocker, followed by centrifugation at 4°C at 14 000 g for 10 min. Supernatant was then collected and incubated with 65µl of protein G agarose (Santa Cruz Biotechnology, Santa Cruz, CA) and 2µg of PKCλ antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and shaken overnight at 4°C. Blanks were incubated with rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) instead of PKCλ antibody. After incubation each tube was centrifuged at 14 000g for 10 minutes. The supernatant was then discarded and the immunoprecipitate washed three times with cold lysis buffer followed by three washes with cold kinase buffer. The remaining pellet represents the PKCλ immunocomplex.

PKCλ activity was determined in total cell lysates using immunoprecipitation and 33P phosphorylation of Ser159-PKCε pseudosubstrate peptide. PKCλ kinase activity was determined by adding 40µl of both kinase buffer (50 mM Tris pH 7.5, 5mM magnesium acetate, 0.1mM sodium orthovanadate, 1mM sodium fluoride, 1mM EGTA, 0.1mM sodium pyrophosphate) (Biomol Research Laboratories, Plymouth Meeting, PA) and reaction buffer (kinase buffer containing 5µg Ser159-PKCε pseudosubstrate peptide 5ul of [γ-33P]ATP(50µCi), aprotinin, leupeptin, okadaic acid) (Sigma, St-Louis, MO) followed by incubation for 30 minutes at 30°C. After incubation, the reaction was terminated by
quenching with 25µl of ice cold quenching solution containing 0.1mM ATP, 100mM EDTA, pH 8 (Sigma, St-Louis, MO). Quantitation of $^{33}$P radioactivity was performed by scintillation. Samples were vortexed and 60µl were spotted onto 2X2cm phosphocellulose papers. Each phosphocellulose paper was then washed four times in 75mM phosphoric acid followed by one wash in 80% ethanol. After air drying, the phosphocellulose papers were placed into scintillation tubes containing 10ml of scintillation fluid (Beckman Industries). Scintillation was measured using a liquid scintillation counter (Beckman LSC5 scintillation counter) set for $^{33}$P detection. Activity was determined by subtracting the count of the IgG blank from the counts of the samples. Data for PKCλ activities are expressed as the percentage of those observed in control.

3.3 STATISTICAL ANALYSIS

All results are presented as mean±standard deviation (SD). Statistical comparisons were made by analysis of variance (ANOVA) followed by Dunn’s multiple contrast test to identify differences between various treatments. Exact p-values are provided for each comparison.

3.4 RESULTS:

3.4.1 Endothelin-1 Attenuates Nitric Oxide Release (Appendix 1-2)

Figure 11 depicts the dose response effect of ET-1 on HSVEC NO production in culture medium following 24 hours of exposure. Basal NO production was unaffected at low concentrations of ET-1 whereas exposure to 1nM of ET-1 significantly increased NO production (two-way ANOVA F= 131.54, p<0.0001). In contrast, higher doses of ET-1 (above 1nM) led to a significant dose-dependent reduction in total nitrite levels (F= 131.54, p<0.0001).
Figure 12 depicts the effect of our treatments on NO production following 30-minute or 24-hour exposure (group*time effect $F= 21.90, p<0.0001$). HSVEC exposed to ET-1 (100 nM) for 30 minutes resulted in a slight but significant reduction in total nitrite levels compared to control (group effect $F= 304.29, p<0.0001$), whereas the 24-hour treatment resulted in a 42% reduction, (group effect $F= 304.29, p<0.0001$). Total nitrite production following ET-1 exposure for 24 hours was significantly lower than total nitrite released after 30 minutes of treatment (time effect $F= 4.73, p<0.03$). BOS treatment attenuated the effect of ET-1 on NO production (group effect $F= 304.29, p<0.0001$). HSVEC exposed to BOS alone demonstrated a significant increase in NO production following 30 minutes of treatment with an increase of approximately 20% after 24 hours compared to control (group effect $F= 304.29, p<0.0001$). Differences in total nitrite production between acute and prolonged BOS exposure were statistically significant (time effect $F= 4.73, p<0.03$).

**3.4.2 Effect of Protein Kinase C on Nitric oxide Production (Appendix 2)**

Exposure to the PKC inhibitors Cal and Chel significantly reduced NO release from HSVECs after 30 minutes with a further reduction after 24 hours (Figure 12) compared to all groups (group*time effect $F=21.90, p<0.0001$). Co-incubation of ET-1 with PKC inhibitors did not demonstrate any synergistic reduction in NO production. HSVEC treated with the PKC agonist, PMA, demonstrated increased NO release compared to control and significantly abrogated the effects of ET-1 following both 30-minute and 24-hour exposure (group effect $F=304.29, p<0.0001$) (Figure 12). PMA exposure demonstrated a time-dependent increase in total NO production between 30-minute and 24-hour exposure (time effect $F= 4.73, p<0.03$).
Isoform specific inhibition (24 hours) was performed to determine the role of each specific isoform in the regulation of NO (Figure 13). PKCλ and PKCe inhibition demonstrated a significant reduction in total nitrite levels compared to control (groups F=304.29, p<0.0001) while PKCδ inhibition did not alter total nitrite production. Co-incubation of ET-1 with ZI or EI resulted in a significant reduction in total nitrite level compared to both control and ET-1 alone (group effect F=304.29, p<0.0001) (Figure 13). Simultaneous exposure to ET-1 and rottlerin significantly attenuated the ET-1-induced reduction in total nitrite production; however, rottlerin failed to restore levels back to normal (group effect F=304.29, p<0.0001) (Figure 13).

3.4.3 Effect of Protein Kinase A on Nitrite Production (Appendix 2)

PKA antagonism with H89 (30 minutes and 24 hours) did not alter total nitrite production compared to control (Figure 14). Co-incubation of H89 with ET resulted in a significant reduction in total nitrite released for HSVEC compared to H89 alone and control (group effect F=304.29, p<0.0001) (Figure 14). A greater decrease in total nitrite levels was seen following 24 hour exposure compared to 30 minutes (time effect F= 4.73, p<0.03). No significant differences were seen between ET-1 exposure and H89+ET treatment. The PKA activator, FOS, had no effect on total nitrite production compared to control. FOS+ET exposure significantly reduced total nitrite levels below control levels but not compared to ET-1. Prolonged treatment with FOS+ET further reduced total nitrite levels (time effect F= 4.73, p<0.03) (Figure 14).

3.4.4 Effect of Protein Kinase B on Nitrate/Nitrite Production (Appendix 2)

Akt/PKB inhibition with SH5 for either 30 minutes or 24 hours resulted in a significant reduction in total nitrite produced by HSVEC compared to control (group effect
F=304.29, p<0.0001) (Figure 15). A time-dependent decrease was also seen with Akt/PKB inhibition (time effect F= 4.73, p<0.03). SH5 exposure for 24 hours led to lower total nitrite levels compared to both control and ET-1. Co-incubation with ET-1 demonstrated a significant additive reduction in total nitrite levels. Prolonged treatment with SH5+ET resulted in an even greater reduction in total nitrite (Figure 15).

3.4.5 Reactive Oxygen Species Formation (Appendix 3)

ROS production following 24 hour exposure to treatment demonstrated significant differences between groups (group effect F=4.16, p<0.0001) (Figure 16-18). HSVEC treated with ET-1 displayed a significant rise in ROS production compared to control. SH5 therapy also increased intracellular ROS formation compared to control but to a lesser extent than ET-1. Combined treatment with SH5 and ET-1 led to a greater increase in ROS production compared to SH5 or ET-1 alone. BOS therapy was able to block ET-1 derived ROS production. No differences were seen between BOS or BOS+ET and control. PKC agonism resulted in ROS production while PKC antagonism reduced free radical formation. PKC inhibition partially attenuated ET-1 induced ROS production while ET-1 increased PMA induced free radical release. Modulation of PKA activity did not demonstrate any significant differences compared to control and did not effect ET-1 driven ROS production.

Isoform specific inhibition of PKC revealed significant differences between groups. ZI (PKC\(\lambda\) inhibitor) and EI (PKC\(\epsilon\) inhibitor) exposure inhibited ROS production while rottlerin (PKC\(\delta\) inhibitor (DI)) did not demonstrate any difference compared to control. Co-incubation of ET-1 with EI or DI (PKC\(\epsilon\) and \(\delta\) inhibitors) resulted in a significant reduction in intracellular ROS production compared to ET-1 alone. Co-incubation with ZI did not lead to any significant changes compared to ET-1 alone.
Antioxidant therapy revealed several differences between groups. In control cells sepiapterin, L-NAME, apocynin and TI did not significantly lower ROS production. TE resulted in a significant 13% reduction in ROS production. The use of sepiapterin, L-NAME and apocynin reduced ET-1 derived ROS, while TI had no significant effect on ET-1 induced ROS production. TE reduced ROS production to the same degree in both control and ET-1 exposed cells indicating that ET-1 derived ROS is not through inhibition of SOD.

3.4.6 Protein Kinase C Isoforms (Appendix 4)

We assessed our cell cultures for the presence of several PKC isoforms (α, β, γ, δ, ε, λ, ι, ζ). Only the α, δ, ε, ι and λ were present in our HSVECs. PKCα was expressed solely on the membrane fraction whereas PKCι was expressed exclusively in the cytosolic fraction. Therefore, we were unable to demonstrate changes in the M/C ratios of these isoforms after treatment. PKCδ, ε, and λ isoforms demonstrated significant changes following treatment (group*time effect PKCδ F=3.62, p<0.0067, group*time effect PKCε F=7.43, p<0.0001, group*time effect PKCλ F=4.44, p<0.0001) (Figure 20-25).

ET-1 exposure for thirty minutes resulted in a significant translocation of both PKCδ and PKCε isoforms to the membrane (group effect PKCδ F=157.49, p<0.0001, group effect PKCε F=45.13, p<0.0001). 24 hour exposure to ET-1 resulted in a further increase in PKCδ and PKCε translocation (time effect PKCδ F=25.14, p<0.0001, time effect PKCε F=60.63, p<0.0001) (Figure 20-23). Examination of the PKCλ isoform demonstrated that ET-1 significantly reduced the M/C ratio (suggesting inhibition of translocation) compared to control (group effect PKCλ F=9.28, p<0.0001) (Figure 24-25).

PKC inhibition with Cal and Chel resulted in a significantly reduced M/C ratio for PKCδ following 30-minute and 24-hour exposure in control and ET-1 treated cells (Figure
PKC inhibition impaired translocation of PKCλ only in the control groups \( (p<0.05) \), whereas translocation of PKCε was reduced only in the ET-1 treated group \( (p<0.05) \). PKC activation with PMA resulted in translocation to the membrane of PKCλ in both the control and ET-1 treatment group \( (p<0.05) \), while only increasing PKCδ and PKCε translocation in the control group.

The effects of BOS on HSVEC PKC translocation were significant. BOS demonstrated the ability to abrogate ET-1 induced effects on PKCδ and PKCε translocation at both 30 minutes and 24 hours \( (\text{group*time effect PKCδ } F=3.62, \ p<0.0067, \ \text{group*time effect PKCε } F=7.43, \ p<0.0001) \) \( (\text{Figure 20-23}) \). HSVEC exposed to BOS with and without ET-1 demonstrated increased translocation of PKCλ compared to control \( (\text{group*time effect PKCλ } F=4.44, \ p<0.0001) \) \( (\text{Figure 24-25}) \).

Following 30-minute exposure to SH5, a significant reduction in PKCλ translocation was seen compared to control with a further decrease seen after co-incubation with ET-1 \( (\text{Figure 15}) \) \( (p<0.05) \). SH5+ET therapy for 24 hours reduced PKCλ translocation compared to control however, showed no differences compared to ET-1 alone. SH5 treatment for 24 hours did not further reduce PKCλ translocation compared to 30 minute exposure \( (\text{Figure 24-25}) \) \( (p<0.05) \). SH5 treatment resulted in no significant changes in PKCδ and PKCε translocation after 30 minutes or 24 hours \( (\text{Figure 11-14}) \). Modulation of PKA activity had no significant effect on ET-1 induced changes in PKC translocation \( (\text{Figure 20-25}) \).

### 3.4.7 Protein Kinase Activity (Appendix 5-7)

#### 3.4.7.1 Total PKC Activity (Appendix 5)

Significant differences in total PKC activity were seen between groups and incubation times \( (\text{group*time } F=23.56, \ p<0.0001) \). ET-1 exposure significantly reduced
PKC activity compared to control after both 30 minutes and 24 hours (Figure 26-27). BOS treatment not only blocked ET-1 induced effects but also resulted in enhanced PKC activity (Figure 26). BOS demonstrated a time-dependent increase in PKC activity.

As expected, HSVEC treated with Cal and Chel demonstrated reduced PKC activity at 30 minutes and a greater reduction in activity following 24 hours of exposure. No synergistic reduction in PKC activity was seen with concomitant ET-1 treatment. PMA exposure increased PKC activity and abrogated ET-1 induced effects (Figure 26). PMA (with or without ET-1) resulted in increased activity compared to all other groups and led to further activation following 24-hour incubation.

Akt/PKB inhibition slightly reduced PKC activity following 30 minutes with no further reduction after 24 hours (Figure 27). Co-incubation with ET-1 resulted in a significant reduction compared to control but not compared to ET-1 alone at 30 minutes or 24 hours (Figure 27).

PKA modulation with either FOS or H89 did not result in any significant changes in PKC activity (Figure 27). Co-incubation with ET-1 led to a similar reduction in PKC activity as with ET-1 alone (Figure 27). Co-incubation for 24 hours further decreased PKC activity compared to 30-minute exposure (Figure 27).

### 3.4.7.2 Isoform specific PKC Activity (Appendix 6)

To confirm that the PKC ZI inhibitor inhibited PKCζ, we measured PKCζ activity specifically (Figure 28). We found that ZI specifically inhibited PKCζ in our endothelial cells and since HSVEC do not express PKCζ, ZI is an isoform specific inhibitor of PKCζ in our model (two-way ANOVA F=53.81, p<0.0001). Overall PKC activation was reduced following ET-1 treatment. Using this assay we assessed whether PKCζ is inhibited
following ET-1 treatment since translocation of atypical PKC isoforms does not necessarily correlate with cellular PKC activity. ET-1 exposed cells demonstrated a significant reduction in PKCλ activity compared to control but PKC activity was significantly higher compared to ZI (F=53.81, p<0.0001). We also confirmed that PMA resulted in significant activation of PKCλ (F=53.81, p<0.0001).

3.4.7.3 PKA Activity (Appendix 5)

No differences between groups were seen in PKA activity (group*time F=0.31, p>0.3) (Figure 29-30). ET-1 exposure regardless of duration did not alter PKA activation compared to control. Modulation of PKC or PKB activity also failed to change cellular PKA activation. HSVEC treated with BOS did not modify PKA activation. As expected, exposure to FOS led to PKA activation while therapy with H89 resulted in decreased activity compared to all other groups (group effect F=107.37 p<0.0001). There was a time-dependent decrease in activity following H89 treatment and a time-dependent increase following FOS exposure (time effect 11.87, p<0.0001) (Figure 30).

3.4.7.4 PKB activation (Appendix 7)

ET-1 exposure for 30 minutes demonstrated a reduction in ser-pAkt phosphorylation compared to control (ser-pAkt/total Akt ratio: group*time F=27.84, p<0.0001) (Figure 31-32). Following incubation for 24 hours with ET-1, ser-pAkt phosphorylation was further reduced (Figure 31-32). Treatment with BOS significantly abrogated the effects of ET-1. Both PKC inhibition and activation for 30 minutes resulted in the greatest reduction in ser-pAkt compared to all groups. PKC modulation for 24 hours further decreased serine phosphorylation (Figure 31-32). Examination of thr-pAkt demonstrated no differences between groups regardless of incubation time except for cells treated with either Cal or Chel
which showed a significant decrease in thr-pAkt following 30 minutes with a greater reduction after 24 hours (thr-pAkt/total Akt ratio: group*time F=6.93, p<0.0001) (Figure 31-32).

### 3.4.8 eNOS, iNOS, Caveolin-1 Expression and eNOS Translocation (Appendix 8)

After 30 minute exposure to ET-1, there was no effect observed on caveolin-1, iNOS and eNOS (caveolin-1: F=0.22, p=0.95) expression in HSVECs. (Figure 33-35). Significant differences in eNOS expression were seen following 24 hour exposure (group*time F=20.94, p<0.0001). ET-1 exposure for 24 hours markedly reduced eNOS protein expression by 46±4% compared to control (Figure 36). Furthermore, PKC antagonism showed a significant reduction in eNOS protein expression compared to control to a level comparable to ET-1 treatment. No interactive effects were seen with PKC inhibitors and ET-1. PKC activation with PMA did result in a significant upregulation of eNOS protein expression compared to control and completely abolished the ET-1 induced reduction of eNOS expression (Figure 36). Figure 36 also demonstrates that BOS treatment prevented ET-1 induced downregulation of eNOS. Expression of both iNOS and caveolin-1 (caveolin-1: F=0.22, p=0.95) were unaffected following 24-hour treatment with ET-1. (Figure 24-25).

Prolonged or acute Akt/PKB inhibition did alter eNOS or iNOS regulation. Co-incubation with ET-1 resulted in a significant eNOS downregulation following 24 hours that was comparable to ET-1 alone. (groups F=19.97, p<0.0001) PKA modulation did not alter eNOS protein expression. Neither PKA nor Akt/PKB modified iNOS or caveolin-1 expression (caveolin-1: F=0.22, p=0.95).

eNOS is more active when it is in the cytosol and not sequestered by the protein caveolin-1 on the membrane. Figures 37-38 displays the eNOS M/C ratio following
treatment. ET-1 resulted in a higher M/C ratio following 30 minute and 24 hour exposure compared to control, Cal and Chel (group*time F=10.89, p<0.0001). BOS treatment abrogated ET-1 sequestration of eNOS at both time points. PKC inhibition resulted in a higher M/C ratio compared to control while PMA resulted in a reduced M/C ratio. Co-incubation of Cal or Chel with ET-1 resulted in a further increase in eNOS M/C ratio compared to Cal or Chel alone (group*time F=10.89, p<0.0001). The PMA+ET group demonstrated a higher M/C ratio than PMA alone (group*time F=10.89, p<0.0001). PKA modulation had no effect on eNOS localization. Akt/PKB inhibition led to a significantly increased M/C ratio following both 30 minutes and 24 hours. Co-incubation of SH5 with ET-1 for 24 hours demonstrated an additive increase in the eNOS M/C ratio compared to control (group*time F=10.89, p<0.0001).

3.5 CONCLUSIONS:

These series of experiments determined the cellular mechanisms involved in ET-1 induced endothelial dysfunction. We specifically, examined the role of PKA, PKB and PKC. Elevated ET-1 levels result in a dose-dependent and time-dependent reduction in cellular NO production while increasing ROS formation. ET-1 induced NO impairment is mediated by PKC and Akt/PKB alterations. We found that ET-1 results in decreased PKC\(\lambda\) translocation leading to decreased NO production. PKC\(\delta\) and PKC\(\varepsilon\) are translocated to the membrane following ET-1 exposure leading to decreased NO bioavailability by shifting eNOS from an NO producer to a ROS producer.

NO production is determined by eNOS expression, localization, conformational state and activity. PKC affects NO production through alterations of all these factors. ET-1 ability to decrease PKC\(\lambda\) translocation and activity results in impaired NO production by increasing
eNOS and caveolin-1 binding over baseline. Under normal condition, PKC\(\lambda\) is involved in maintaining a steady state eNOS M/C ratio therefore, decreased PKC\(\lambda\) translocation and activity results in increased eNOS M/C ratio and reduced NO production. In addition, decreased PKC\(\lambda\) activity reduces NO production by decreasing eNOS expression which will reduce overall production of NO by two mechanisms. First, with reduced eNOS expression there will be less NO formation. Second, reduced eNOS expression in the presence of normal caveolin-1 expression results in a proportionally greater eNOS sequestration. PKC\(\lambda\) inhibition also decreases ROS production demonstrating that PKC\(\lambda\) inhibition also reduces NO production by decreasing eNOS activity. ET-1 increased PKC\(\delta\) translocation and activity leads to impaired NO production by uncoupling eNOS. This finding is supported by our data that shows PKC\(\delta\) inhibition in control cells has no effect on NO production while PKC\(\delta\) activation lead to NO impairment while increasing ROS production. Therefore, PKC\(\delta\) alters eNOS by uncoupling the enzyme. The effect of PKC\(\epsilon\) translocation and activation results in eNOS activation as seen by increased NO and ROS production. Therefore, the ultimate result of PKC\(\epsilon\) translocation and activation depends on eNOS expression, and functional state. Since, ET-1 results in decreased eNOS expression and functional uncoupling, PKC\(\epsilon\) results in a proportionally greater increase in ROS production than NO formation. This shift also contributes to decreased NO production as increased ROS formation quenches NO.

Akt/PKB and PKA have been implicated in NO regulation in various cell types. In these series of experiments we examined the role of Akt/PKB and PKA in HSVEC exposed to ET-1. ET-1 demonstrated the ability to inhibit Akt/PKB which decreases NO formation. ET-1 exposure did not alter PKA activity. Furthermore, we made the observation that PKA
modulation does not alter NO regulation in HSVEC. We also observed that PKA does not regulate NO homeostasis and ROS production. Our data indicated that ET-1 reduces NO homeostasis by downregulating eNOS. Akt/PKB does not alter eNOS expression however, it increases eNOS M/C ratio. Therefore, ET-1 localizes eNOS to the membrane where it is less active therefore impairing NO homeostasis through Akt/PKB inhibition.

Our investigations demonstrated that ET-1 induced ROS production is mediated through eNOS uncoupling and NADPH oxidase. This is supported by the ability of sepiapterin and apocynin to block ET-1 driven ROS generation. Therefore, ET-1 results in eNOS uncoupling and NADPH oxidase activation. The formation of ROS can also be driven by the induction and activation of iNOS. We did not find any significant induction of iNOS in our treatment groups. Our data showed that BOS therapy protects against the deleterious effects of ET-1 on HSVEC. BOS has the ability to increase NO production from baseline. This effect is likely mediated by the ability of BOS to reduce ROS production and increase PKC\(\lambda\) activity leading to increased NO formation.

In conclusion, the results of these series of experiments suggest that ET-1 exposure results in inhibition of PKC\(\lambda\) translocation and activity as well as increased PKC delta and epsilon translocation. In addition, ET-1 results in an inhibition of Akt/PKB activity. These changes result in decreased eNOS expression, increased binding of eNOS to membrane-bound caveolin and a shift towards the production of ROS instead of NO formation. Ultimately, ET-1 impairs NO homeostasis through PKC and PKB not via PKA.
CHAPTER FOUR

THE INTERACTIVE EFFECTS OF HYPOXIA/REPERFUSION AND ENDOThELIN-1 ON ENDOThELIAL FUNCTION, VIABILITY AND SURVIVAL: THE ROLE OF PROTEIN KINASES
4.1 INTRODUCTION

Primary allograft dysfunction and CAV are the principal causes of early and late morbidity and mortality following cardiac transplantation. Endothelial dysfunction is associated with the development of transplant coronary artery disease and primary cardiac allograft dysfunction [563-568;581-585;935]. I/R injury and ET-1 are important players in the pathogenesis of endothelial dysfunction following cardiac transplantation. We have previously demonstrated that ET-1 reduces cardiac allograft function and survival [17]. In this study we also showed that intermittent perfusion of the cardiac allograft with donor blood improves myocardial and endothelial function. This observation indicates that H/R has less deleterious effects to the endothelium than I/R. The interactive effects of H/R and ET-1 remain unknown. Hollenberg et al and Marti et al found that the severity of endothelial dysfunction is correlated with the degree of intimal thickening [566-568]. Verrier et al. observed in aortic allografts that I/R results in endothelial injury leading to the development of transplant vasculopathy [8]. Therefore, blocking ET-1 as well as limiting I/R injury may reduce the development of primary graft failure and allograft vasculopathy.

Changes in signal transduction pathways such as PKC play important roles in ischemic injury. Mochly-Rosen et al have demonstrated that PKCe activation and PKCδ inhibition is cardioprotective following cardiac transplantation [166-168]. Therefore, PKC modulation and ET-1 antagonism improves cardiomyocyte function and survival following cardiac transplantation. The benefits of such treatment on endothelial cells have yet to be fully elucidated. Akt/PKB plays an important role in cell survival in several cell types. The effect of H/R as well as ET-1 on human endothelial cell Akt/PKB remains to be assessed.

Our investigations aim to determine the interactive effects of H/R and ET-1 on
endothelial cell function and viability. We also explored the consequences of H/R instead of I/R on human endothelial cell protein kinase modulation and cell death. We finally, assessed whether our observations documented during normoxia conditions continue to exist after hypoxia. We therefore made the following hypothesis:

1) Elevated levels of ET-1 exacerbate the effects of H/R on endothelial dysfunction and death.

2) H/R injury is mediated through apoptosis, necrosis and autophagy.

4) Elevated ET-1 levels increase apoptosis in HSVEC by increasing caspase activation.

4) H/R and ET-1 alters protein kinase activity (PKC, PKB and PKA) activity leading to endothelial dysfunction, altered NO and ROS production as well as increased membrane bound eNOS over cytosolic eNOS.

5) ET-1 exacerbates H/R induced ROS production which leads to cell death.

6) H/R and ET-1 ROS formation is due to eNOS uncoupling and NADPH oxidase activation.

7) Antioxidant therapy will improve cell survival.

8) We also propose that BOS can prevent ET-1 and H/R induced injury while restoring normal vascular homeostasis.

9) Isoform specific PKC inhibition alters cell survival as well as NO and ROS formation.
4.2 METHODS

4.2.1 Simulated Hypoxia and Reperfusion

4.2.1.1 Hypoxia System

1. Three-shelf chamber (BioSpherix, Redfield, New York)
2. ProOX, oxygen controller (Model 110, BioSpherix)
3. ProCO2, CO₂ controller (Model 120, BioSpherix)
4. N₂ cylinder and regulator
5. CO₂ cylinder and regulator

4.2.1.2 Hypoxia And Reperfusion Procedure

HSVEC were stabilized by incubation under normoxic conditions for 30 minutes with fresh medium (DMEM+10%FBS) at 21% O₂ at varying supernatant volumes: 200µl (96-well plates, 10 000 cells/plate), 2ml (6-well plates, 50 000 cells/plate) or 8ml (10cm culture plates, 500 000 cells/plate). While stabilization occurred, the set points on the ProOx and ProCO₂ controller were set at 0.1% O₂ and 5.0% CO₂. Following stabilization at normoxic conditions, the medium in each wells were replaced with the treated medium of interest (80µl (96-well plates), 750µl (6-well plates), 2ml (10cm culture plates)) and the plates were placed into the hypoxia chamber and incubated for 24 hours. After hypoxic exposure, the culture medium in each well or plate was replaced with fresh medium (with or without treatment) and cells were incubated under normoxic conditions for the required length of time (reperfusion for 1 hour or 24 hours).

4.2.2 Cell Viability Assay

Cell viability was assessed by using the XTT-II kit (Roche Diagnostics, Canada). After treatment, endothelial cells were incubated with the XTT dye in medium (1:4)
(DMEM+10%FBS) for four hours at 37°C. During the incubation period XTT is converted to formazan by normally functioning mitochondria in viable cells producing a color change. The color change was measured spectrophotometrically at an absorbance wavelength of 450 nm versus a reference wavelength of 620 nm. Sample absorbance values were corrected by subtracting the absorbance of the blanks wells. Comparisons between groups were performed with values calculated by the following equation (100 * corrected sample hypoxic absorbance values ÷ corrected normoxic absorbance value).

4.2.3 CELL DEATH

4.2.3.1 Homogeneous Caspase Assay

A homogeneous caspase assay was performed using a commercially available activity kit (Roche Diagnostics, Canada). Black 96-well plates (Costar, cat # CS003603) were used for these experiments. The homogeneous caspase assay detects principally caspase 3 activity as well as caspase 6 and 7 activation. Activated caspase cleaves the non-fluorescent Asp-Glu-Val-Asp-Rhodamine 110 (DEVD-R110) into the fluorophore Rhodamine 110 (R110) which can be detected using a fluorescence reader.

10,000 cells were seeded into each well two days prior to the experimental protocols. Cells were exposed to the desired treatment as well as to the required hypoxic protocol. The assay reagents were prepared prior to each study. 1ml of substrate stock solution which contains 500μM DEVD-R110 was diluted 1:10 with 9ml of incubation buffer and protected from light. Positive controls were prepared by diluting 25μl of cellular lysate (apoptotic U937 cells treated with 4μg/ml camptothecin) with 225μl of incubation buffer. Standards were prepared by using the R110 standard provided by the manufacture and protected from light. Zvad (benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone) (10μM) (Roche
Diagnostics, Penzberg, Germany) which exhibits a broad specificity for caspase-family proteases was used as our caspase inhibitor.

100µl of medium was added to each of two blank wells (negative control). 100µl of the positive control solution was added to each of two positive control wells. 100µl of each of the standard solutions were added into the appropriate wells in duplicate. Then 100µl of the substrate working solution was added to all 96 wells. Following addition of the substrate working solution, the 96-well plates were incubated at 37°C for two hours. After incubation, plates were read by using a fluorescence plate reader (CytoFluor multi-well plate reader Series 4000) (Excitation = 485nm and emission = 535nm). Sample R110 fluorescence values were corrected by subtracting the R110 fluorescence of the blanks wells. Comparisons between groups were performed on values obtained by the following equation (100 * corrected sample hypoxic fluorescence value ÷ corrected normoxic fluorescence value).

4.2.3.2 Propidium Iodide Labeling

Assessment of necrosis was performed by propidium iodide (PI) labeling. After treatment, cells were stained with 200µl of PI 1:100 (Sigma, St-Louis, MO) for 30 minutes at room temperature. After staining, each well was washed three times with PBS containing Ca^{2+} and magnesium and read in a fluorescent plate reader (CytoFluor multi-well plate reader Series 4000) at an excitation wavelength of 530nm and emission of 620nm. Sample PI fluorescence values were corrected by subtracting the PI fluorescence of the blank wells. Comparisons between groups were performed on values obtained by the following equation (100 * corrected sample hypoxic fluorescence ÷ corrected normoxic fluorescence value).
4.2.3.3 Autophagy

Following hypoxia and reperfusion, cells were incubated with 0.05mM monodansylcadaverine (MDC) (Molecular Probes, Eugene, OR) in 200µl PBS containing Ca\(^{2+}\) and magnesium for 15 minutes at 37°C. Cells were washed three times with PBS and fluorescence measured with a fluorescence plate reader (CytoFluor multi-well plate reader Series 4000) (excitation wavelength of 380nM and emission at 525nM). Sample MDC fluorescence values were corrected by subtracting the MDC fluorescence of the blanks wells. Comparisons between groups were performed on values obtained with the following equation (100 * corrected sample hypoxic fluorescence ÷ corrected normoxic fluorescence).

4.2.3.4 Caspase 8 And 9 Methodology

Caspase 8 and 9 activity was performed by seeding 500,000 cells into 6-well dishes. Cells were exposed to the desired treatment (24 hr of normoxia or 24 hr of hypoxia followed by either 1hr or 24hr of reperfusion). Three wells were used for appropriate positive and negative controls as well as for unlabelled control. At the end of the experimental procedure, the culture medium was aspirated from each well and cells washed with 3ml of PBS. Once washed, 500µl of trypsin solution was added to each well and incubated at 37°C for two minutes. 1ml of medium per well was added to block trypsin activity. Cells were collected and transferred to labeled microtubes. Each tube was centrifuged at room temperature at 3000rpm for five minutes. After centrifugation, the supernatant was aspirated and the pellets were re-suspended gently with 300µl of medium. 1µl of the appropriate caspase substrate was added (Red-IEDT-FMK for caspase-8 or FITC-LEDH-FMK for caspase-9 (Calbiochem, San Diego, California). Cells were vortexed very gently and incubated with the lids open at 37°C for one hour. Following incubation, the cells were centrifuged as above
and the supernatant aspirated. 500µl of wash buffer (Calbiochem, San Diego, California) was added and the cells were re-suspended and centrifuged. The wash buffer was removed and discarded. This step was repeated twice. Following the final wash the cells were re-suspended in 300µl of wash buffer and transferred to the flow cytometer tubes. Each tube was placed on ice and protect from light. FACS scan analysis was then performed (Becton Dickinson, Franklin Lakes, New Jersey).

**4.2.4 Reactive Oxygen Species Formation**

ROS production was detected as previously described in chapter three. Measurements were performed at four different time points. 1) After normoxic exposure. 2) Hypoxic exposure. 3) Hypoxia and 1-hour reperfusion. 4) Hypoxia and 24-hour reperfusion.

**4.2.5 NO Production**

Total nitrite and nitrite production was measured as previously described in chapter three. Nitrite levels were measured at four different time points. 1) After normoxic exposure. 2) Hypoxic exposure. 3) Hypoxia and 1-hour reperfusion. 4) Hypoxia and 24-hour reperfusion.

**4.2.6 Western Blot Analysis**

Western Blot analysis (eNOS, iNOS, PKCδ,ε,λ, Akt/PKB, β-actin and caveolin-1) was performed as previously described in chapter three. Analyses were performed at four different time points. 1) After normoxic exposure. 2) Hypoxic exposure. 3) Hypoxia and 1-hour reperfusion. 4) Hypoxia and 24-hour reperfusion.
4.2.7 PKC/PKA Activity

PKC and PKA activity and assessed as previously described in chapter three. Assessments were performed at four different time points. 1) After normoxic exposure. 2) Hypoxic exposure. 3) Hypoxia and 1-hour reperfusion. 4) Hypoxia and 24-hour reperfusion.

4.3 STATISTICAL ANALYSIS

All results are presented as mean±SD. Statistical comparisons were made by analysis of variance (ANOVA) followed by Dunn’s multiple contrast test to identify differences between various treatments. Exact p-values are provided for each comparison.

4.4 RESULTS

4.4.1 Cell Viability Analysis (Appendix 9)

Significant differences in cell viability were observed between groups (group*time effect F= 40.80, p<0.0001) (Figure 39-42). A significant increase in XTT absorbance was seen in the normoxic group incubated for 48 hours compared to 24 hours. H/R, our control group, significantly reduced cell viability compared to normoxia following both 4 hours and 24 hours of reperfusion (Figure 39). Exposure to ET-1 during hypoxia further impaired cell viability compared to control at both time points. BOS therapy during hypoxia resulted in a significant protection against both H/R and ET-1 exposure at 4 hours and 24 hours of reperfusion. BOS treatment during hypoxia restored viability to normoxic levels following 4 hours of reperfusion but not after 24 hours. PKC modulation during hypoxia demonstrated that PKC inhibition or activation reduced viability compared to control after 4 hours of reperfusion with a further decrease after 24 hours (Figure 40). Co-incubation of Cal, Chel or PMA with ET-1 led to a further impairment in cell viability compared to control, Cal, Chel or PMA (Figure 40). Altering PKA activity had no significant impact on H/R and ET-1
induced effects on cell viability. Inhibition of Akt/PKB with SH5 reduced viability compared to control (4 and 24 hours) and ET-1 (24 hours) (Figure 41). ET-1 co-incubated with SH5 further impaired viability compared to SH5 at 4 hours but not after 24 hours. SH5 treatment with or without ET-1 resulted in a massive reduction in viability after 24 hours. Inhibition of apoptosis with the caspase inhibitor zvad during hypoxia demonstrated no protective effects in the control group at 4 hours but improved viability after 24 hours. Zvad limited the added effect of ET-1 at both time points (Figure 39). When zvad was given during hypoxia and reperfusion viability was restored to 100% for both control and ET-1 at 4 and 24 hours of reperfusion.

Treatment with BOS during reperfusion had significant beneficial effects on cell viability (Figure 42). BOS given after hypoxia restored viability to near normoxic levels. ET-1 treatment during hypoxia followed by BOS therapy at reperfusion improved cell viability and prevented the decline in viability seen after 24 hours. When BOS was added to the medium during both hypoxia and reperfusion cell viability was maintained (Figure 42). Extreme reduction in viability was seen following ET-1 exposure during hypoxia and reperfusion to the lowest levels of all groups. BOS was able to partially attenuate SH5 induced impairment and completely block the added effect of ET-1 on SH5.

4.4.2 Cell Death

4.4.2.1 Apoptosis

4.4.2.1.1 Homogenous Caspase Activity (Appendix 10-13)

Induction of apoptosis following the experimental protocols revealed significant differences between groups (group*time effect F= 44.34, p<0.0001) (Figure 5-14). Exposure to hypoxia for 24 hours resulted in a significant 121% increase in caspase activity compared
to normoxia (Figure 43). Reperfusion further increased caspase activation in a time-dependent manner (Figure 43). ET-1 exposure during hypoxia increased apoptosis compared to control following hypoxia alone and reperfusion (1hr and 24hr) (Figure 43). Treatment with BOS limited both hypoxic and ET-1 effects on caspase activity (Figure 43).

When the apoptosis inhibitor zvad was given to the control and ET-1 groups during hypoxia it prevented caspase activation following hypoxia but failed to prevent caspase activation during reperfusion. Zvad therapy during both hypoxia and reperfusion inhibited caspase activation throughout H/R. The exposure of hydrogen peroxide (25μM) during hypoxia greatly enhanced apoptosis after H/R (Figure 44). Hydrogen peroxide treatment during both hypoxia and reperfusion resulted in the greatest activation of caspase activity.

Exposure of HSVEC to Cal or Chel during hypoxia increased apoptosis compared to control at all time points (Figure 45). PMA treatment during hypoxia increased caspase activation following H/R. Co-incubation of PKC inhibitors or PMA with ET-1 further increased caspase activation compared to Cal, Chel and PMA. Pharmacological modulation of PKA activity did not demonstrate any effects beyond those of H/R alone. In addition, PKA modulation did not alter ET-1 induced effects. Inhibition of Akt/PKB resulted in apoptosis that was significantly greater than control and all treatment groups (Figure 46). SH5 treatment showed a time dependent increase in caspase activity (Figure 46). Similar to PKC modulation, SH5 co-incubated with ET-1 revealed an additive increase in caspase activation (Figure 46).

Isoform specific inhibition of PKCλ during hypoxia significant increased caspase activation compare to H/R alone while rottlerin and EI significant reduced apoptosis during all time points (Figure 47). ZI incubation during hypoxia and reperfusion showed increased
caspase activity compared to control after one hour of reperfusion with no differences at 24 hours (Figure 47). Rottlerin and EI therapy during hypoxia significantly reduced ET-1 induced caspase activation following both hypoxia and reperfusion. Further protection against the development of apoptosis was seen when rottlerin and EI were given during hypoxia and reperfusion. ZI and ET-1 co-incubation during hypoxia or H/R resulted in additive increase in caspase activity.

Antioxidant therapy significantly reduced apoptosis (Figure 48-51). Treatment with antioxidants (sepiapterin, L-NAME, Apocynin, TE, and TI) during hypoxia alone reduced apoptosis compared to control following hypoxia and reperfusion. However, greater reduction in caspase activity was seen when antioxidant treatment was given during both hypoxia and reperfusion. Antioxidant therapy limited but did not abolish apoptosis. In addition, a time dependent increase in caspase activity was also seen in antioxidant treated control groups. Similarly, ET-1 exposed groups that received antioxidant treatment during hypoxia demonstrated a significant decrease in apoptosis compared to ET-1 but not compared to control. Following 24 hours of reperfusion sepiapterin and L-NAME showed a greater caspase reduction compared to apocynin, TE and TI. Antioxidant treatment during hypoxia extended protective effects during reperfusion. Groups exposed to ET-1 and antioxidant treatment during hypoxia and reperfusion revealed additional reduction in apoptosis compared to hypoxic therapy alone. Sepiapterin and L-NAME demonstrated that same superior protection compared to apocynin, TE and TI. BOS treatment in addition to antioxidant therapy offered an approximate 10% decrease in apoptosis compared to BOS alone.
Hypoxia followed by reperfusion with BOS demonstrated a significant reduction in caspase activity (Figure 52). Greater protection was seen when BOS was given throughout hypoxia and reperfusion (Figure 52). A significant increase in apoptosis was seen when ET-1 was added to the reperfusion medium. BOS treatment was also able to limit apoptosis in the group that received SH5 with or without ET-1 during hypoxia.

4.4.2.1.2 Caspase 8 And 9 Activation (Appendix 14-17)

Analysis of caspase 8 and 9 activation revealed significant differences between groups (caspase 8 groups*time effect F= 23.18, p<0.0001) (caspase 9 groups*time effect F= 21.19, p<0.0001) (Figure 53-60). Following hypoxia and reperfusion there was a significant time dependent increase in caspase 8 and 9 activation (Figure 53, 57). ET-1 treatment during hypoxia further increased caspase 8 and 9 activation after hypoxia and reperfusion (Figure 53, 56). Exposure to ET-1 during hypoxia and reperfusion greatly enhanced caspase 8 and 9 driven apoptosis compared to all groups (Figure 56, 60).

Therapy with BOS during hypoxia abrogated ET-1 induced caspase 8 and 9 activation following hypoxia and reperfusion (Figure 53, 57). BOS exposure during hypoxia limited caspase 8 and 9 activation in the control group (Figure 53, 57). Hypoxia and reperfusion treatment with BOS demonstrated the greatest benefit in preventing caspase 8 and 9 activation (Figure 56, 60).

PKC inhibition and activation during hypoxia resulted in caspase 8 and 9 activation after hypoxia and reperfusion for one and 24 hours (Figure 54, 58). Co-incubation of Cal, Chel or PMA with ET-1 resulted in an additive increase in activity (caspase 8 and 9) after hypoxia and reperfusion (1 and 24 hours) (Figure 54, 58). PKA modulation did not alter hypoxia and reperfusion or ET-1 induced caspase 8 or 9 activation (Figure 55, 59).
Akt/PKB inhibition with SH5 during hypoxia caused significant time dependent caspase 8 and 9 activation. In addition, an additive increase was seen with ET-1 co-incubation. BOS treatment during reperfusion limited SH5 induced apoptosis. Inhibition with the apoptosis inhibitor zvad during hypoxia limited caspase 8 and 9 activation during hypoxia and reperfusion (Figure 53, 56). Zvad exposure during both hypoxia and reperfusion blocked caspase 8 and 9 activation (Figure 53, 56).

4.4.2.1.3 Autophagy (Appendix 18)

The formation of autophagosomes is the hallmark of autophagy. Akt/PKB inhibition is known to increase the formation of autophagosomes through mTOR inhibition. Therefore, SH5 was used as our positive control and did demonstrate increased MDC fluorescence compared to all groups (groups*time effect F= 1.04, p>0.4; groups effect F= 109.92, p<0.0001; Time effect F= 1.27, p>0.4) (Figure 61). No induction of autophagy was observed between our treatment (except for SH5) groups during normoxia or following H/R.

4.4.2.1.4 Necrosis (Appendix 19)

Assessment of necrosis demonstrated no significant differences between groups in PI labeling under normoxic conditions (groups*time effect F= 0.04, p>0.9; groups effect F= 1.09, p>0.4; Time effect F= 0.76, p>0.5) (Figure 62). In addition, following H/R no differences were observed between groups. In order to confirm the ability of PI to label DNA, nuclear lysate was used as a positive control and demonstrated significant PI labeling and fluorescence. There was a trend towards increased necrosis with increasing reperfusion.
4.4.3 Nitric Oxide Production (*Appendix 20-21*)

Following H/R all groups demonstrated a significant reduction in total nitrite production compared to its normoxic counterpart (groups*time effect \(F= 8.82, p<0.0001\)) (*Figure 63-66*). H/R significantly reduced total nitrite production compared to normoxia. ET-1 exposure during hypoxia further impaired NO production compared to control (*Figure 63*). When HSVEC were treated with ET-1 during hypoxia and reperfusion a greater reduction in total nitrite levels were seen (*Figure 65*). BOS therapy during hypoxia attenuated both hypoxia and ET-1 induced NO impairment (*Figure 65*). This protection was extended to the reperfusion period even though BOS was not present in the reperfusion medium (*Figure 65*). However, the protective effect of BOS was partially reduced following 24 hours of reperfusion. BOS supplementation during reperfusion further limited the effects of H/R and ET-1 (*Figure 65*). BOS during reperfusion also abrogated the effects of Akt/PKB inhibition during hypoxia (*Figure 65*). PKC inhibition with Cal or Chel during hypoxia resulted in a significant reduction in total nitrite levels after hypoxia and reperfusion compared to control and ET-1 (*Figure 63*). PMA exposure during hypoxia also demonstrated decreased NO production after hypoxia and reperfusion however, the total nitrite levels were the highest compare to all groups (*Figure 63*) therefore PMA exposure attenuated the loss of NO production after H/R. PKA modulation did not alter H/R and ET-1 induced changes in NO production (*Figure 64*). SH5 therapy during hypoxia resulted in lower NO production compared to control and ET-1 (*Figure 64*).

Isoform specific modulation of PKC activity demonstrated significant differences between groups (*Figure 66*). ZI exposure during hypoxia significantly worsened the H/R induced reduction in total nitrite levels in both the control and ET-1 groups. Treatment with
ZI during hypoxia and reperfusion further decreased NO production (Figure 66). Rottlerin treatment during hypoxia in the control group did not alter NO production however, when given during reperfusion NO production was enhanced. In the ET-1 exposed group, PKCδ inhibition with rottlerin during hypoxia demonstrated protective effects against NO impairment with further improvement when rottlerin was given throughout reperfusion (Figure 66). The exposure of HSVEC to ET during hypoxia caused a significant reduction in total nitrite levels in both the control and ET-1 groups. Treatment during hypoxia and reperfusion resulted in further NO impairment (Figure 66).

4.4.4 Reactive Oxygen Species (Appendix 22-24)

ROS assessment following H/R with or without treatment revealed in significant differences between groups (groups*time effect F= 96.67, p<0.0001) (Figure 67-71). Exposure to 24 hours of hypoxia resulted in a small significant increase in ROS production while reperfusion demonstrated a time dependent rise in ROS release (Figure 67). ET-1 exposure enhanced both the effects of hypoxia and reperfusion (Figure 67). A reperfusion time dependent increased in ROS formation was also seen. ET-1 treatment during both hypoxia and reperfusion further increased free radical formation compared to both control and ET-1 during hypoxia alone (Figure 67-68). BOS therapy during hypoxia protected against H/R and ET-1 induced free radical release. The protective effects of BOS also extended to the reperfusion phase. Greater reduction in ROS production was seen when BOS was given during both hypoxia and reperfusion. BOS exposure during reperfusion alone limited the effects of H/R and hypoxic treatment with ET-1 and SH5 groups (Figure 68). PKC inhibition resulted in a significant reduction of free radical formation while activation with PMA increased ROS production following H/R (Figure 67). Co-incubation
of ET-1 with Cal, Chel, and PMA resulted in increased ROS production compared to Cal, Chel and PMA alone (Figure 67). PKA modulation did not affect H/R and ET-1 induced ROS release (Figure 67). SH5 treatment during hypoxia was found to increase free radical formation following both hypoxia and reperfusion. Antioxidant therapy significantly reduced ROS production following H/R (Figure 69-70). When antioxidant therapy was given during hypoxia alone a significant reduction in ROS levels were seen in the reperfusion phase. However, when treatment was continued into the reperfusion phase enhanced protection was seen (Figure 69). TE and TI’s protective effects were diminished at 24 hours of reperfusion when given only during hypoxia. Sepiapterin, L-NAME and apocynin demonstrated greater protection against the formation of free radicals compare to TE and TI. In the ET-1 exposed group antioxidant treatment during hypoxia limited both the effects of hypoxia and ET-1. The benefits of antioxidant treatment during hypoxia also extended to reperfusion in the ET-1 group. The supplementation of antioxidants during reperfusion further reduced the ROS enhancing effects of ET-1 and H/R (Figure 70).

Isoform specific PKC inhibition altered ROS formation following H/R (Figure 71). ZI and EI exposure during hypoxia resulted in significant ROS reduction after hypoxia and reperfusion while DI lowered free radical formation only during reperfusion. In the ET-1 exposed groups, all isoform specific inhibitor treatment during hypoxia decreased ROS production and when therapy was extended during reperfusion a further reduction was observed (Figure 71).

4.4.5 PKC And PKA Activity (Appendix 25)

We observed significant differences in consensus PKC activity following H/R (group*time F=23.56, p<0.0001) (Figure 72). Hypoxia slightly raises PKC activity.
Reperfusion for both one and 24 hours increased PKC activity compared to normoxia and hypoxia. ET-1 treatment during hypoxia slightly reduced consensus activity compared to hypoxia alone however the activity is higher than ET-1 during normoxia (Figure 72). BOS treatment prevented the rise in PKC activity seen in the control group during H/R (Figure 72). PKA activity following H/R did not demonstrate any differences compared to normoxia (Figure 73). ET-1 and BOS exposure during hypoxia also did not alter PKA activation (Figure 73).

**4.4.6 Akt/PKB Phosphorylation (Appendix 26)**

H/R significantly altered Akt/PKB phosphorylation (Serine/Total ratio: group*time F=39.06, p<0.0001) (Threonine/Total ratio: group*time F=21.05, p<0.0001) (Figure 74-76). H/R significantly reduced serine phosphorylation compared to normoxia (Figure 74-75). There was a reperfusion time dependent increase in serine phosphorylation however the serine/total ratios were lower than normoxia. When ET-1 was added during hypoxia a further reduction in serine phosphorylation was seen. The recovery of serine Akt phosphorylation was impaired in all ET-1 exposed groups compared to control (Figure 74-75). BOS treatment during hypoxia limited the effect of hypoxia and one hour of reperfusion in both the control and ET-1 groups. Following 24 hours of reperfusion BOS abrogated ET-1 induced effects. PKC inhibition significantly reduced Akt/PKB serine phosphorylation following hypoxia and reperfusion (Figure 74-75). Co-incubation with ET-1 further lowered serine/total ratio. PMA decreased serine phosphorylation compared to control following 24 hours of reperfusion. An additive effect was seen with ET-1 co-incubation during hypoxia and reperfusion (Figure 74-75).
Significant differences in threonine phosphorylation was observed (Threonine/Total ratio: group*time F=21.05) (Figure 74, 76). Threonine/Total ratio was significantly reduced following hypoxia and one hour of reperfusion compared to normoxia (Figure 74, 76). A return to normoxic threonine phosphorylation was seen after 24 hours of reperfusion. ET-1 treatment and PKC inhibition resulted in lower threonine phosphorylation compared to all other groups following hypoxia and reperfusion. Exposure to PMA resulted in decreased Akt threonine phosphorylation following hypoxia and one hour of reperfusion compared to normoxia with no differences compared to control. BOS treatment during hypoxia significantly abrogated the effects of H/R and ET-1 during hypoxia and reperfusion. BOS demonstrated a slight increase in threonine phosphorylation after 24 hours of reperfusion.

4.4.7 eNOS, iNOS And Caveolin-1 Protein Expression (Appendix 27-28)

eNOS protein expression was significantly altered by H/R (group*time F=48.71, p<0.0001) (Figure 77-78). H/R significantly reduced eNOS expression following hypoxia compared to normoxia (Figure 77). ET-1 treatment during hypoxia further downregulated eNOS expression. BOS abrogated both hypoxia and ET-1 induced alterations in eNOS expression. During reperfusion eNOS expression increased and returned to normoxic levels after 24 hours. Akt/PKB and PKA modulation did not alter the effects of hypoxia or ET-1 on eNOS protein expression (Figure 77). PKC inhibition revealed a significant eNOS downregulation following hypoxia compare to control while PMA resulted in a slight upregulation. eNOS protein expression increased during reperfusion in the Cal and Chel groups back to normoxic levels (Figure 77). iNOS expression was not induced following hypoxia and reperfusion and no differences between groups were seen (Figure 79).
Caveolin-1 expression was unaffected by H/R and by our treatment groups (group*time F=0.20, p>0.9, groups F=0.26, p>0.9, time effect F=0.46 p>0.9) (Figure 80-81).

4.4.8 PKC And eNOS Translocation (Appendix 29-32)

PKC translocation was significantly altered by H/R. (PKCδ group*time effect F=8.67, p<0.0001; PKCε group*time effect F=12.16, p<0.0001; PKCλ group*time effect F=10.45, p<0.0001) (Figure 82-87). Following hypoxia a significantly increased M/C ratio for both PKCδ and PKCε was observed while PKCλ demonstrated a significant decrease compared to normoxia (Figure 82-87). PKCδ membrane translocation was further increased at one hour of reperfusion with a reduction in membrane PKCδ at 24 hours to an M/C ratio that remained higher than normoxia. PKCε demonstrated a gradual reduction in the M/C ratio with reperfusion while PKCλ revealed a gradual increase. PKCε translocation returned to normoxic levels at 24 hours of reperfusion. The PKCε M/C ratios at 24 hours however were still higher, in the PMA groups, than that of normoxia. After 24 hours of reperfusion PKCλ M/C returned to near normoxic levels. The exposure to ET-1 during hypoxia increased delta and epsilon translocation to the membrane while PKCλ showed a decreased M/C ratio compared to control (Figure 82-87). BOS treatment abrogated both hypoxia and ET-1 induced changes in PKC translocation of all three isoforms. PKC inhibition limited hypoxia induced PKCδ and PKCε translocation to the membrane while having no effect on PKCλ M/C ratio (Figure 82-87). PMA exposure attenuated the effects of hypoxia on PKCλ while increasing PKCε translocation and having no effect on PKCδ (Figure 82-87). PKA modulation did not significantly alter PKC translocation following H/R compared to control. Co-incubation with ET-1 demonstrated a similar PKC isoform translocation profile as with ET-1 alone. Akt/PKB inhibition did not alter PKCδ or PKCε translocation over hypoxic
effects alone (Figure 82-87). The PKC\(\lambda\) M/C ratio was slightly reduced during hypoxia following SH5 exposure. Co-incubation with ET-1 showed M/C ratios that were similar to ET-1 exposure (Figure 82-87).

eNOS localization was significantly affected by H/R (group*time F=8.52, p<0.0001) (Figure 88-89). Hypoxia significantly increased the eNOS M/C ratio with a return to the normoxic ratio following one and 24 hours of reperfusion (Figure 88-89). ET-1 treatment during hypoxia further localized eNOS to the membrane after hypoxia with a return to normal following reperfusion. BOS therapy limited the effects of hypoxia on eNOS translocation (Figure 88-89). Akt/PKB inhibition during hypoxia demonstrated a higher M/C ratio compared to control with an additive increase in eNOS localization when co-incubated with ET-1 (Figure 88-89). PKC inhibition during hypoxia increased the M/C ratio compared to control with a further increase with ET-1 co-incubation. PMA exposure revealed eNOS localization to the membrane following hypoxia and a greater membrane presence of eNOS when co-incubated with ET-1 (Figure 88-89). All groups demonstrated a return to a normoxic M/C ratio following one or 24 hours of reperfusion (Figure 88-89).

4.5 CONCLUSIONS

This study examined the effects of H/R on cell death. We have observed the role of protein kinases (PKA, PKB, and PKC) in H/R effects. The potential protective effects of BOS were also assessed. We have demonstrated that H/R results in cell death through the activation of apoptosis and necrosis not via autophagy. H/R increases ROS formation and impairs NO production. Our data showed that ROS leads to apoptosis. The use of antioxidants further confirmed our results that ROS lead to cell death. PKC as well as
Akt/PKB played a role in H/R induced cell death, NO impairment and ROS production while PKA did not alter H/R effects.

Our investigations revealed that PKC delta and epsilon translocation occurs following hypoxia while a decreased PKC\(\lambda\) M/C ratio was observed. Isoform specific inhibition of these isoforms during hypoxia revealed that PKC\(\delta\) and PKC\(\varepsilon\) activation leads to apoptotic cell death while PKC\(\lambda\) inhibition results in apoptosis. The inhibition of Akt/PKB leads to apoptosis. Following H/R, decreased Akt/PKB phosphorylation was also observed. Therefore, H/R causes apoptosis through the activation of PKC delta and epsilon as well as by the inhibition of PKC\(\lambda\) and Akt/PKB. ET-1 is also involved in H/R induced effects as demonstrated by the protective effects of BOS.

ET-1 exposure during hypoxia exacerbated the effect of H/R. We observed further NO impairment, increased ROS production and greater apoptosis with ET-1 treatment. These effects were achieved through increased PKC\(\delta\) and PKC\(\varepsilon\) translocation while further decreasing PKC\(\lambda\) and Akt/PKB activity compared to H/R alone. BOS therapy during hypoxia was able to attenuate both ET-1 and hypoxic effects. BOS also demonstrated PC like properties as the protective effects were extended to the reperfusion phase where BOS was not present. Antioxidant therapy also limited ET-1 and H/R induced ROS production and cell death. Our investigation showed that eNOS and NADPH oxidase are key players in ET-1 as well as H/R induced ROS formation.

Reperfusion resulted in greater ROS formation and increased cell death. The presence of ET-1 during reperfusion increased ROS production and cell death. BOS treatment during H/R offered greater protected against ROS formation and cell death. Treatment with antioxidants during reperfusion resulted in decreased ROS production as
well as cell death following H/R alone and in the ET-1 exposed group. PKC delta, epsilon and lambda also play a role in reperfusion. Our study showed that all our isoform specific PKC inhibitors led to decreased ROS production during reperfusion.

The results of our investigations suggest that ET-1 worsens hypoxic and reperfusion injury though the alteration of isoform specific modulation of PKC and inhibition of Akt/PKB. We also show that BOS can reduce the deleterious effects of both H/R and ET-1 as well as provide PC like protection. The protective effects of BOS during hypoxia also indicate the role of ET-1 in hypoxic injury and cell death. Defining the mechanisms by which H/R and ET-1 result in cell injury and death may allow for pharmacologic strategies to protect the endothelium.
CHAPTER FIVE

IMMUNOSUPPRESSIVE DRUG INDUCED VASOMOTOR DYSFUNCTION:
THE ROLE OF NITRIC OXIDE AND ENDOTHELIN-1 REGULATION
5.1 INTRODUCTION:

CyA was the first anti-rejection drug that impacted the results of clinical organ transplantation by reducing the incidence and severity of rejection and remains an important component of modern therapy. Unfortunately, CyA is associated with many side effects [749;753;948;949] and has also been implicated in the development of endothelial dysfunction and transplant vasculopathy [594;717;728;749-753]. CyA can influence CAV by increasing plasma lipid concentrations, causing hypertension or by direct injury to the endothelium [753-756]. It is believed that the endothelial injury induced by CyA results in impaired vascular homeostasis and transplant coronary disease.

The mechanisms by which CyA results in endothelial dysfunction are not fully elucidated. However, CyA is known to impair vascular vasodilation [719;722-724] and may induce vasoconstriction [725-727]. Potential mechanisms include alterations in NO – ET-1 regulation. Impaired NO homeostasis may be a result of decreases in mRNA or protein expression of eNOS in CyA treated patients. Several investigators have demonstrated that eNOS mRNA expression is increased after CyA treatment [950] suggesting that impaired NO production is likely due to decreases in eNOS protein synthesis or a shift to free radical production [730]. There is also evidence that CyA may generate free radicals [728]. These free radicals may result in direct endothelial injury and impaired vasomotor function [723;728-730]. Gupta et al showed that BOS can attenuate oxidative stress in an experimental model of I/R [918]. Yamaguchi et al observed, in a rodent model, the importance of ET-1 blockade in preventing graft coronary artery disease [103].
Several studies have demonstrated that SRL has both an anti-proliferative effect and a protective effect against the development of CAV in a rodent model [750;774]. Corbin et al have shown that SRL led to vasomotor relaxation of rat aortic rings in a dose-dependent fashion [775]. In contrast, Jeanmart et al demonstrated that SRL results in worse endothelial dependent vasorelaxation than CyA [715]. However, both of these studies exposed the vasculature to SRL ex vivo for a short duration making in vivo correlation unreliable. In addition the vehicle used for SRL in Jeanmart’s study also resulted in endothelial dysfunction [715;775]. Whether SRL results in endothelial dysfunction or impairs NO- ET-1 homeostasis remains unclear. Clinically, SRL has been demonstrated to reduce the incidence, progression and severity of CAV [781], yet the mechanisms by which SRL leads to endothelial protection and prevention of CAV remains unknown.

Our investigations assess the role of CyA and SRL on the development of endothelial dysfunction in a rodent model of vascular injury. Specifically, we examine the effects of CyA and SRL exposure on NO homeostasis and ET-1 signaling. We chose a non-transplant model for these initial experiments in order to evaluate the direct effects of CyA and SRL in the absence of either I/R or immune-mediated injury. We also hypothesized that BH4 and BOS therapy may prevent CyA induced endothelial dysfunction by improving NO production and limiting free radical injury.

5.2 METHODS:

Animal care conformed to the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals. (NIH publication no 86-23, revised 1996) Male Lewis rats (200-300g, n=8 per group) were administered the drug of interest (saline control,
CyA (5mg/kg/day) (Novartis, Canada), SRL (1.5mg/kg/day) (Wyeth Pharmaceuticals, Canada), BOS (100mg), BH₄ (7.5mg) (Sigma, St-Louis, MO), BOS+BH₄, BOS+CyA, BH₄+CyA, or BOS+BH₄+CyA) via peritoneal injection for a period of fourteen days prior to assessment of endothelial function. On the day of sacrifice (day 15), rats were anaesthetized using 2% isoflurane in a sealed chamber and anaesthesia maintained with a face mask (A.D.S 1000 ventilator, Engler Engineering Corporation). Median sternotomy was then performed and the heart excised for myocardial analysis and segments of thoracic aorta (TAo) procured for assessment of endothelial function. Segments of the descending thoracic aorta were procured for biochemical assessment. Prior to tissue excision, 2cc of blood from the right ventricle was collected for analysis of ET-1 and immunosuppressive drug levels. The rats were then exsanguinated under general anesthesia.

### 5.2.1 Endothelial Function Assessment

Endothelial dependent and independent vascular relaxation was assessed in isolated segments of thoracic aorta following treatment. The aorta was dissected and segments 5 mm in length were employed for the assessment of in-vitro vascular function using a small vessel myograft for isometric tension recording. After mounting the vessel on a pressure transducer, maximum vasoconstriction was achieved with exposure to phenylephrine (100nM) (Sigma, St-Louis, Mo). Following stabilization, endothelial dependent relaxation was assessed by incremental exposure to Ach (10nM – 10µM) (Sigma, St-Louis, Mo). Endothelial independent relaxation was assessed using incremental exposure to SNP (10nM – 5µM) (Sigma, St-Louis, Mo). Complete vasomotor data for all groups is presented in the figures in order to visualize the dose-dependent effects of each intervention. In addition, Emax% was calculated by determining the % maximal relaxation from phenylephrine-
induced vasoconstriction. ED$_{50}$, defined as the concentration required to achieve half-maximum vasorelaxation, was compared between groups. Following SNP washout, sensitivity to vasospasm was assessed by incremental exposure to ET-1 (0.05nM – 10 nM) (Sigma, St-Louis, Mo) and %Cmax calculated as the maximum increase in tension from baseline. Each animal yielded two aortic segments. Data were included if the variability between segments was less than 10% and data averaged to yield one result per animal.

5.2.2 Plasma Measurements

5.2.2.1 ET Levels

Venous blood was aspirated from the right ventricle prior to exsanguination. Blood samples were centrifuged (14 000rpm) in order to collect the plasma fraction which was then snap frozen in liquid nitrogen. Plasma ET-1 was extracted using C$_{18}$ Sep-Pack columns following acidification with 1% trifluoroacetic acid. ET-1 was extracted by adding an equal volume of plasma (200µl) to a 1% trifluoroacetic acid (TFA) buffer and incubated for 10 minutes on ice. The samples were centrifuged at 4ºC for 20 minutes at 13000 rpm. Plasma samples were placed through the C$_{18}$ Sep-Pack columns using an extraction manifold at a rate of 4-5 seconds per drop. The columns were washed three times with TFA buffer and the samples were re-run through the columns. Following another three washes, a 60% acetonitrile - 1% TFA buffer was added to the column and the sample collected. The acetonitrile was evaporated with N$_2$ using the evaporation manifold. Samples were then frozen in liquid N$_2$ and frozen-dried overnight.

ET-1 was quantified by using a commercial ELISA assay (Biomedica, Vienna, Austria). Extracted ET-1 was re-constituted with assay buffer on ice. 200µl of re-constituted samples were added to each well of the microtiter plates followed by the
addition of 50µl of detection (ET-1) antibody. The microtiter plates were shaken and incubated overnight at room temperature. After incubation, the content of the wells were discarded, washed five times and 200µl of conjugate was added. The microtiter plates were placed in a dry bath incubator at 37ºC for three hours. The contents of the wells were discarded and the wells washed five times. 200µl of substrate was added to each well for 30 minutes at room temperature. After 30 minutes, 50µl of stop solution was added and absorption was immediately read by an ELISA reader at a wavelength of 405nm against a reference of 630nm (µQuant Universal Microplate Spectrophotometer, Bio-Tek Instruments, Vermont, USA). Software used for analysis was the KC4 software form Bio-Tek instruments.

5.2.2.2 CyA and SRL Levels

CyA and SRL levels were obtained from all treated animals on day of sacrifice. Under anesthesia, blood was taken from the right ventricle in EDTA tubes. CyA and SRL levels (in nanograms per milliliter) were determined by the Toronto Medical Laboratories by high pressure liquid chromatography.

5.2.3 Assessment of Oxidative Injury

8-isoprostane levels were measured as an indicator of free radical injury [951]. 8-isoprostane is the stable end product of arachidonic acid oxidation generated by ROS injury. Determination of 8-isoprostane levels from thoracic aortic tissue was performed using a commercially available kit (Cayman Chemical Company; Ann Arbor, MI).

Descending aortic tissue was weighed prior to homogenization. 10µl of 10mM phosphate buffer (PB; containing 0.1M K₂HPO₄, 0.1M KH₂PO₄, and NaCl at a pH of 7.4) was added to each homogenization tube per 1mg of tissue. 0.5% butylated hydroxy
Toluene (BHT) was supplemented to each tube (20µl per ml of PB). Tissues were homogenized on ice. Homogenates were transferred into eppendorf tubes with equal volumes of 15% KOH and incubated at 40°C for 60 minutes. Following incubation each sample was centrifuged at 4 000rpm for 10 minutes at 4°C. Supernatants were transferred into 15ml tubes containing 2ml of eicosanoid affinity column buffer (containing 1M K$_2$HPO$_4$ and 1M KH$_2$PO$_4$) to achieve a pH of 7.2. The supernatants were passed through the affinity column twice. Using an elution buffer (95% ethanol solution) the eluant was collected into microcentrifuge tubes. The samples were diluted 1:10 in enzyme immunoassay buffer (EIAB). 8-isoprostane levels were determined by enzyme immunoassay. Samples were added to a 96-well NSB plate and incubated 1:1:1 with AchE tracer and antiserum for 18 hours at room temperature. Wells were washed five times and both the tracer and Ellman solution were added to the plate for 90 minutes of developing. Absorption was immediately read by an ELISA reader at a wavelength of 405nm against a reference of 630nm (µQuant Universal Microplate Spectrophotometer, Bio-Tek Instruments, Vermont, USA). The KC4 software from Bio-Tek instruments was used for analysis.

Baseline assessments were made on aortic segments harvested from control animals not subjected to IP injections and the %change from these baseline values was calculated to compare differences between groups.

5.2.4 Reactive Oxygen Species Formation

The formation of ROS was detected by loading cultured HSVEC with the fluoroprobe H$_2$DCFDA (Molecular Probes, Eugene, OR) as discussed in chapter three.
ROS analysis was performed in 96-well cultures. PBS (Con), CyA (50ng/ml), SRL (10ng/ml), BOS, BOS+CyA were added to each well and incubated at 37°C for 24 hours. At the end of treatment ROS production was measured by fluorescence at an excitation wavelength at 485nM and emission of 535nM (CytoFluor multi-well plate reader Series 4000, PerSeptive Biosystem) using CytoFluor software analysis version 4.2.1. Sample absorbance values were corrected by subtracting the absorbance from the blanks wells. Comparisons between groups were performed with the following equation (100 * corrected treated group fluorescence values / corrected control fluorescence value).

5.2.5 Western Blot Analysis

Thoracic aortas and left ventricular (LV) biopsies were immediately collected after harvesting and snap frozen in liquid nitrogen until analyzed. TAos were used for eNOS, iNOS, ET_A and ET_B receptor expression and LV biopsies were assessed for TNF-α and TGF-β expression. The collected tissues were homogenized at 4°C and prepared for analysis. Protein determination was determined by the method described by Bradford [947].

Western Blot determined protein expression of iNOS, and eNOS, with the use of protein specific monoclonal antibodies (Biosciences; Mississauga, Canada), TNF-α, TGF-β, and ET_A and ET_B receptor with the use of protein specific polyclonal antibodies (Chemicon; Temecula, CA). Samples were separated using 4% stacking and 10% running tris-glycine SDS-PAGE gels. Gels were then transferred onto PVDF membranes. Blocking was performed with buffer for 1 hour at room temperature and then monoclonal IgG at a dilution of 1:2500 was reacted with the blots for 12 hours at 4°C. Incubation with secondary antibody was then performed for 1 hour after washing. Comparisons between groups were
performed using computerized densitometric analysis with a commercially available software program (Quality one, BioRad, California). TAo densitometric data were corrected for β-actin expression while LV data were corrected for GAPDH expression. No differences in β-actin and GAPDH protein expression were seen between groups at any time point.

5.3 STATISTICAL ANALYSIS

Statistical analysis was performed with the SAS statistical software program version 8.2 (SAS institute, Inc, Cary, NC). Continuous data were analyzed by analysis of variance (ANOVA) and are expressed as the mean ± SD. When the F-statistic of the ANOVA was significant (p<0.05), a Duncan’s multiple range test was performed to specify differences between groups.

5.4 RESULTS:

All animals survived until day of sacrifice with no complications. There was minimal variability from aortic segments within each animal and no animals were excluded from the study. Plasma measurements of each immunosuppressant were within the normal clinical range (Appendix 33).

5.4.1 Endothelial Function (Appendix 34)

Endothelial dependent vasorelaxation of the thoracic aorta was impaired following CyA treatment (Figure 90-93). Figure 90-93 depicts the cumulative dose-response curves to Ach in aortic segments following 2-week exposure to treatment. A significant interactive effect was seen between group and Ach concentration (group*dose effect F= 3.08, p<0.0001) CyA treated animals displayed a significantly impaired response to Ach when compared to SRL and control. SRL demonstrated increased vasodilatory response than control at every Ach concentration. BOS therapy restored a
normal Ach dose-response in CyA treated rats. BH₄ therapy limited CyA induced vasomotor impairment but failed to normalize the response to Ach to control levels. BOS+BH₄ treated groups demonstrated improved endothelial dependent vasodilation compared to control. Significant differences were also seen between groups at maximal endothelial dependent vasodilation (Emax%). CyA resulted in an Emax% of 24±7%, lower than that of control (52±8%) and SRL (58±4%) (Figure 94). BH₄ therapy for CyA treated animals normalized endothelial dependent vasodilation to Ach (Figure 94). BOS treatment demonstrated similar protective effects to the endothelium compared to BH₄ (Figure 94). Combined therapy with BOS and BH₄ revealed significantly enhanced vasodilatory properties compared to control (Figure 94). Another measure of endothelial function is the ED₅₀ for Ach (Figure 95). Exposure to CyA resulted in a significant increase in the Ach ED₅₀ (3.2x10⁻⁸±0.3mole/L) compared to control (2.0x10⁻⁸±0.5mole/L) (group effect F=12.74, p<0.0001) (Figure 95). SRL had an ED₅₀ for Ach that was significantly lower than all treated groups (Figure 95). Treatment with BH₄ improved the ED₅₀ for Ach in CyA exposed rats but did not restore it to normal, while BOS therapy (with or without CyA) significantly reduced the ED₅₀ for Ach compared to control (Figure 95). Simultaneous ET-1 antagonism with NO augmentation (BOS+BH₄) also enhanced the endothelial dependent vasodilatory properties of the vasculature compared to control (Figure 95).

Endothelial independent vasorelaxation of the thoracic aorta was significantly impaired following CyA treatment (Figure 96-99). A significant interactive effect was seen between group and SNP concentration (group*dose effect F=2.53, p<0.0001). Figure 96-99 demonstrates that at low concentrations of SNP, CyA (CyA alone,
BH₄+CyA and BOS+CyA) treated animals had an impaired vasodilatory response compared to control. BOS+BH₄+CyA did not show the same lag in SNP response as did the other CyA exposed rats (Figure 96-99). Significant differences in maximal endothelial independent vasorelaxation to SNP were seen between groups. No differences were seen between CyA, SRL and control. BOS or BH₄ therapy to CyA exposed rats improved Emax% back to near control levels (Figure 100). The BH₄ alone and BOS+BH₄ groups (with and without CyA) had higher Emax% compared to control. When examining the relaxation curves, a lag in vasodilatory response to SNP is observed following CyA therapy. The concentration of SNP necessary to achieve 50% of maximal vasodilatory response demonstrated significant differences between groups (Figure 101) (F=24.84, p<0.0001). A doubling of the SNP ED₅₀ was seen after CyA treatment (6.3x10⁻⁸±1.2mole/L) compared to both control (3.2x10⁻⁸±1.0mole/L) and SRL (2.5x10⁻⁸±1.1mole/L) indicating dysfunction (group effect F= 24.84, p<0.0001). Differences between SRL and control were not significant. Furthermore, BH₄ treatment for CyA treated animals limited the increased ED₅₀ for SNP seen following CyA exposure but failed to restore the ED₅₀ to control levels. BH₄ treatment alone resulted in a significantly lower ED₅₀ than that of control animals. BOS therapy completely abrogated CyA effects on SNP induced vasodilation and the BOS alone group had an ED₅₀ concentration lower than that of control animals. Combined treatment with both BOS and BH₄ (with or without CyA) resulted in the lowest ED₅₀ compared to all groups.

Sensitivity to ET-1 induced vasospasm revealed significant differences between groups (Figure 102-105). Figures 102-105 depict the cumulative dose-response curves for ET-1 and demonstrated significant differences between groups (group*dose effect
F=9.27, p<0.0001). CyA treatment significantly increased vasospasm compared to control and SRL. BH$_4$ treated groups resulted in increased vasospasm compared to all treatment groups except for CyA (Figure 102-105). All BOS treated groups showed a dose-response curve that was significantly lower than that of control and BOS was able to abrogate both CyA and BH$_4$ induced vasospasm (Figure 102-105). %Cmax was greater in the CyA treated group compared to control and SRL (CyA: 259%±3% vs. SRL: 230±5% vs. control: 211±8%, (Figure 106). BH$_4$ exposure significantly increased %Cmax (Figure 106). BOS therapy lowered %Cmax and was able to abrogate the effects of CyA and BH$_4$ (Figure 106). The ET-1 ED$_{50}$ (the concentration of ET-1 required to elicit half-maximal vasoconstriction) was significantly different between groups. Compared to control (2.51x10$^{-9}$±0.3mole/L), CyA exposure resulted in a significantly lower ED$_{50}$ (1.78x10$^{-9}$±0.1mole/L) indicating increased sensitivity to ET-1-induced vasospasm (group effect F=16.76, p<0.0001) (Figure 107). SRL treated animals did not show any differences versus control (Figure 107). BH$_4$+CyA resulted in the lowest ED$_{50}$ compared to all groups indicating heightened sensitivity (Figure 107). The BH$_4$ exposed group had an ET-1 ED$_{50}$ lower than that of control while BOS and combined therapy had higher ED$_{50}$ compared to all treated groups (Figure 107).

**5.4.2 Plasma ET-1 Levels (Appendix 33)**

CyA exposure did not alter plasma ET-1 levels compared to control (CyA 0.9±0.1 fmol/L; control 1.0±0.1 fmol/L) (Figure 108). However, SRL treated animals demonstrated a significantly lower plasma ET-1 level (0.4±0.1 fmol/L) (group effect F= 25.73, p<0.0001) (Figure 10). BH$_4$ and BH$_4$+CyA treatment resulted in an approximate 50% reduction of ET-1 levels compared to both control and CyA treated animals (Figure
10). BOS exposure also reduced plasma ET-1 levels (Figure 108). Control animals had an ET-1 plasma concentration which was in the reported normal physiological range [726].

5.4.3 Oxidative Injury (Appendix 33)

CyA treatment resulted in oxidative injury as measured by 8-isoprostane levels compared to control and SRL (CyA: 50%±2% vs. SRL: 3%±3% vs. control: 2%±5%, p=0.05) (group effect F=73.52, p<0.0001). BH₄ treated animals had reduced 8-isoprostane levels compared to control (Figure 109). CyA animals exposed to BH₄ demonstrated lower oxidative stress than controls (Figure 109). Exposure to BOS prevented CyA-induced ROS injury to the vasculature (Figure 109). All animals exposed to BH₄ had reduced 8-isoprostane production compared to control. Combination therapy with BOS and BH₄ did not demonstrate any additive benefit (Figure 109). Oxidative injury was not likely a result of an increased inflammatory response as myocardial protein expression of both TNF-α and TGF-β were similar between groups (See section 5.4.7).

5.4.4 ROS Cell Data (Appendix 33)

Free radical production was significantly increased in cell cultures following CyA exposure compared to control and SRL (group effect F=56.74, p<0.0001) (Figure 110). CyA treated cells exposed to BOS abrogated ROS generation to near control levels. ROS production was reduced below control levels in BOS treated cell cultures (Figure 110). We have previously demonstrated that BH₄ abrogated CyA-induced free radical production (unpublished data (Figure 110)).
5.4.5 Endothelin Receptor Expression (Appendix 35)

Thoracic aortic ET<sub>A</sub> receptor protein expression was upregulated by 33%±5% following CyA exposure compared to both control and SRL (group effect F= 21.04, p<0.0001) (Figure 111). CyA exposed animals that received BOS or BH<sub>4</sub> therapy had ET<sub>A</sub> receptor expression that was similar to control. Animals with combined exposure to BOS+BH<sub>4</sub> also had ET<sub>A</sub> receptor expression in the control range (Figure 111). ET<sub>B</sub> receptor protein expression was not significantly different between control, CyA and SRL treated animals (Figure 112). However, BH<sub>4</sub> treatment (with or without CyA exposure) resulted in a 20-35% upregulation in ET<sub>B</sub> receptor expression compared to all other experimental groups (group effect F= 9.62, p<0.0001) (Figure 112). BOS exposure did not alter ET<sub>B</sub> receptor protein expression compared to control and completely abrogated BH<sub>4</sub> induced upregulation (Figure 112).

5.4.6 Nitric Oxide Synthase Expression (Appendix 35)

Two week exposure to CyA resulted in a 28%±4% downregulation of eNOS protein expression compared to both control and SRL (group effect F= 80.59, p<0.0001) (Figure 113). CyA treatment had the lowest eNOS expression compared to all groups (Figure 113). While BH<sub>4</sub> therapy attenuated the CyA induced reduction in eNOS protein expression (10%±3% downregulation), it did not normalize it to control levels (Figure 113). BOS treatment also partially abrogated CyA-induced eNOS downregulation (Figure 113). Combined therapy with BOS and BH<sub>4</sub> normalized CyA-induced eNOS downregulation to control levels while BOS alone and BOS+BH<sub>4</sub> treated animals had a significant upregulation in eNOS expression (Figure 113).
Extremely low iNOS expression was observed in all animals, with no differences between groups (group effect F= 0.15, p>0.8) (Figure 113).

5.4.7 Pro-Inflammatory Cytokine Expression (Appendix 35)

Myocardial protein expression of both TNF-α and TGF-β did not differ between groups (TNF-α: F value= 1.77, p>0.22) (TGF-β: F value= 0.88, p>0.5) (Figure 114-115).

5.5 CONCLUSIONS:

This study examined the effect of the immunosuppressants CyA and SRL on NO and ET-1 homeostasis (summarized in table 2). We also looked at the effects of NO augmentation and ET-1 antagonism. We determined that CyA alters both NO and ET-1 regulation leading to impairment in vasodilation, increased vasospasm and increased ROS injury. A two-week course of CyA impaired both endothelial dependent and independent vasodilation. This dysfunction was marked by eNOS downregulation, ET_Α receptor upregulation and free radical injury. CyA did not alter ET-1 plasma levels and therefore, in the presence of NO impairment, CyA causes a relative increase in plasma ET-1 levels.

NO augmentation demonstrated the ability to attenuate the deleterious effects of CyA on vasomotor function. BH_4 treatment improved vasomotor function, normalized eNOS protein expression and prevented ROS injury. NO augmentation with BH_4 also led to heightened vasoreactivity to ET-1. BH_4 exposure demonstrated a significant reduction in ET-1 levels. BOS therapy showed vasomotor protective effects against CyA-induced dysfunction. The protective effect included improved vasodilation and reduced sensitivity to vasospasm. BOS also possessed the ability to abrogate BH_4 induced vasospasm. In addition, animals treated with BOS had reduced ROS injury and decreased ET-1 plasma levels.
Our data revealed that SRL therapy lacks the deleterious effects of CyA on the vasculature. SRL preserves NO homeostasis and maintains eNOS and \( \text{ET}_A \) receptor expression at near normal levels. SRL also decreased ET-1 plasma levels. SRL did not demonstrate free radical injury to the vasculature. The ability of SRL to decrease ET-1 levels may be one potential mechanism by which it reduces the development and severity of CAV.

These findings provide important mechanistic data to explain CyA induced injury and the development of CAV. Our investigations strongly suggest a potential clinical role for simultaneous ET-1 antagonism and NO augmentation or the substitution of SRL for those patients receiving CyA.
CHAPTER SIX

DISCUSSION
6.1 INTRODUCTION

Endothelial dysfunction is a key player in several pathological diseases [24;25;573;580;594;599;600;720;952-961]. They include atherosclerosis and CAV [24;25;573;580;594;599;600;720;952;953;956;959-961]. Impairment in NO homeostasis is a hallmark of endothelial dysfunction and is responsible for many of its deleterious consequences [962-964]. Recently, ET-1 has been implicated in the development of endothelial dysfunction as well as other pathologies such as heart failure, atherosclerosis, CAV and I/R injury [15;17;33;601;602;965-972]. Many conditions result in elevated ET-1 levels such as heart failure and ischemia reperfusion, MI or cardiac transplantation [15;33;587;608;747;868;909;925;971]. Several investigators have demonstrated the deleterious effects of ET-1 on cardiomyocytes and the potential beneficial effects of ET-1 antagonism on preventing myocardial ischemic injury [17;918;919;971-974]. The role of ET-1 in endothelial dysfunction has been less extensively studied. Originally, the role of ET-1 in the endothelial cell was described as increasing NO production however, recent evidence indicates that elevated ET-1 levels impair NO homeostasis leading to endothelial dysfunction [607;609]. It remains controversial whether ET-1 increases or impairs endothelial cell NO production. Furthermore, the mechanisms behind the effects of ET-1 on NO production have not been fully elucidated in human endothelial cells. We hypothesized that elevated ET-1 levels impairs NO homeostasis leading to the development of atherosclerosis and CAV.

Heart failure remains the only cardiovascular diagnosis increasing in incidence [1;2]. Heart failure is also associated with elevated ET-1 levels and its negative effects. ET-1 antagonism limits ET-1 deleterious consequences however, the ENABLE trials
(Endothelin Antagonist Bosentan for Lowering Cardiac Events in Heart Failure) demonstrated a higher rate of sudden death, potentially due to hypotension related arrhythmias. Therefore, determining the mechanism of benefit for ET-1 blockade may permit the development of novel targeted therapies without the side effects associated with direct ET-1 antagonism.

CAV has several known etiological factors including I/R, ET-1 and immunosuppressive drug injury. Verrier et al showed that I/R can lead to the development of CAV [8]. Therefore, limiting the injury during allograft storage and transplantation may reduce the development of CAV. ET-1 has been correlated with the development, severity and progression of CAV while ET-1 antagonism has been shown to decrease the development of CAV [33;103;564;586;601;603;605;608;748;925-928]. Elucidating the mechanisms behind ET-1 induced injury may lead to a better understanding of the pathogenesis of CAV. Immunosuppressive drugs have also been implicated in the development of CAV. Several investigator have demonstrated the CyA leads to endothelial dysfunction and CAV [594;711-721]. We aimed to demonstrate that CyA leads to endothelial dysfunction by altering NO and ET-1 homeostasis. We also intended to show that BOS and NO augmentation will prevent endothelial injury. In addition we have compared CyA to SRL a class of immunosuppressive drug that is associated with decreased CAV.

Understanding the mechanisms behind ET-1 and H/R dysfunction may lead to improved outcomes in the cardiovascular patient and following cardiac transplantation. Several investigators have demonstrated the role of PKC in ischemic injury[416-421]. Mochly-Rosen et al showed that PKC isoform specific changes can lead to protection or
injury of the cardiomyocyte following ischemia [166-168;407]. Akt/PKB and PKA have been implicated in NO homeostasis [301;504]. Akt/PKB is also involved in ischemia reperfusion injury [544-561]. We hypothesized that PKA, PKB and PKC are involved in endothelial cell NO and ROS production as well as hypoxic and reperfusion injury.

We demonstrated in our series of investigations that ET-1, H/R and CyA result in endothelial dysfunction as characterized by deceased NO production and increased ROS generation. We also showed that H/R leads to apoptotic cell death and that H/R induced injury in mediated partially by ET-1 production. Our data revealed that PKC as well as PKB play critical roles in both ET-1 and H/R induced endothelial cell injury. ET-1 antagonism appeared to protect the endothelium from both ET-1 and H/R induced injury. ET-1 antagonism with BOS also protected endothelial function in animals subjected to CyA therapy. In addition, BOS demonstrated PC like properties as it protect against reperfusion injury even when given during hypoxia alone. We found that ET-1 is a key player in endothelial dysfunction and H/R induced injury mediating its effects by alterations in PKC and PKB activity resulting in impaired NO production, increased ROS generation and cell death.

6.2 CELL CULTURE MODEL

HSVECs were used in our cellular experiments. Endothelial cell were easily identified in our plate by light microscopy as oval shaped (15X20µm) cells. The use of an endothelial cell culture model to assess the potential response of the endothelium to various stimuli has been well established.

Simulated hypoxia and reperfusion models have been previously used [23]. Our model attempts to mimics the in vivo transplant model we have previously studied
In this model the cardiac allograft is exposed to donor blood during storage therefore effectively changing the conditions from I/R to H/R. To render the endothelial cell hypoxic we decreased the oxygen tension to 0.1% representing a PO$_2$ of less than 8mmHg. We chose a low oxygen tension to elicit a significant injury to the endothelial cell. Endothelial cells do not become hypoxic until the partial pressure of oxygen decreases to below 30mmHg. Our design employed a sufficiently low oxygen tension in order to ensure that hypoxia resulted in cellular injury and enabled us to document the protective effects of BOS. We also reduced the volume of the culture medium during hypoxia to more closely represent the allograft perfusion model. In this model the endothelial cell milieu accumulates with toxic metabolites and displays a decrease in pH. Furthermore, the perfusate pH is also decreased with time. During reperfusion the culture medium is changed and volume restored to prehypoxic levels. This is done to mimic reperfusion whereby the toxic metabolites are washed out and nutrients become available for the endothelial cells. We believe that our in vitro H/R technique represents a close surrogate to the in vivo allograft perfusion model.

6.3 ANIMAL MODEL

Lewis rats were injected with the desired treatment for a two-week course. Rodent models for immunosuppressive drug studies have been extensively used in the past and provides the ability to perform several biochemical and tissue analyses that cannot be performed clinically [723;727;740;742]. Although not a transplant model, the present study was designed to investigate the direct effects of CyA and SRL on vascular function and the protective effects of BOS and BH$_4$ in the absence of an immune response or a period of I/R as seen following transplantation. Clearly, the effects of the latter two variables will need further assessment in a heterotopic transplant model similar to that of Poston and
Yamaguchi [103;779]. However, prior to completing these studies, it was important to document the direct effects of these agents on recipient endothelium. Thus, we deliberately chose to employ a non-transplant model and evaluated the vascular effects directly associated with CyA and SRL exposure without the confounding factors associated with transplantation.

6.4 THE ROLE OF PROTEIN KINASES IN ENDOTHELIN-1 INDUCED ENDOTHELIAL DYSFUNCTION: ALTERED NITRIC OXIDE AND FREE RADICAL REGULATION

ET-1 plays an important role in normal vessel homeostasis and is associated with adverse outcomes following cardiac surgery [17;563;603-605]. Endothelial dysfunction is implicated in the development of coronary vasospasm, unstable angina, myocardial infarction, atherosclerosis, and transplant coronary disease [33;564;600;603;608;975]. The normally functioning endothelium maintains ET-1 and NO in balance. Impairment in NO production and/or increased release of ET-1 are key initiators of endothelial dysfunction and injury [604]. Given the importance of a normally functioning endothelium we sought to determine the mechanisms by which high levels of ET-1 impairs NO bioavailability in isolated human endothelial cells.

We have made the following observations:

1. Isolated HSVECs exposed to ET-1 demonstrate a dose-dependent change in NO production. At levels above 1nM there is a significant dose-dependent reduction in cellular NO production.

2. ROS production is enhanced by ET-1 treatment through eNOS uncoupling and NADPH oxidase and not through iNOS induction.

3. ET-1 blockade with BOS can maintain cellular NO production.
4. PKC inhibition leads to a severe reduction in cellular NO production with no synergistic effect observed with simultaneous exposure to ET-1.

5. PKC stimulation with PMA significantly increases cellular NO production and is capable of completing reversing the inhibitory effects of ET-1 exposure.

6. ET-1 appears to selectively stimulate PKC$\delta$ and $\varepsilon$ translocation, while inhibiting $\lambda$ translocation.

7. ET-1 exposure results in decreased eNOS expression and an increase in the membrane to cytosolic ratio, suggesting increased binding to membrane-bound caveolin-1.

8. Akt/PKB phosphorylation is reduced following ET-1 exposure.

9. Akt/PKB inhibition reduces NO production and increases the eNOS M/C ratio.

10. Akt/PKB inhibition selectively inhibits PKC$\lambda$ translocation.

6.4.1 EFFECTS OF ET-1 ON NO AND ROS PRODUCTION

Our study demonstrated that ET-1 has a concentration-dependent effect on endothelial cell NO production. Elevated levels of ET-1 resulted in a dose-dependent decrease in NO bioavailability. Although several investigators have reported ET-1 induced changes in NO production, it remains unclear if physiologic levels of ET-1 increase or decrease NO bioavailability [607;609;976]. Elegant studies by Spinale et al have demonstrated a four-fold increase in myocardial interstitial ET-1 levels following cardiopulmonary bypass [977]. However, even at peak levels postoperatively, myocardial ET-1 levels were only 50 fmol/L and in plasma these levels were below 20 fmol/L. At doses of 100 pmol/L, we observed no effect of ET-1 on cellular NO production. 1 nmol/L resulted
in higher NO production while 10 nmol/L inhibited NO production. As in previous studies, a dose of 100 nmol/L was chosen to completely bind both the ETA and ETB receptors [934].

ET-1 also induced a time-dependent decrease in NO production. As seen in our study, 30 minutes of exposure decreased NO levels compared to control with a greater reduction following 24 hours. Elevated levels of ET-1 generated ROS which can have several detrimental effects to the cardiac allograft. Our data supports the hypothesis that ET-1 derived ROS is mediated through eNOS uncoupling and NADPH oxidase. L-NAME inhibition of ET-1 induced ROS production demonstrated that eNOS played an important role in free radical formation. The ability of sepiapterin to reduce ET-1 ROS release revealed that eNOS uncoupling is a mechanism by which ET-1 generates ROS. Since L-NAME and sepiapterin did not fully block the effects of ET-1, we investigated the possibility that another pathway of ROS production was involved. ET-1 was found to also produce ROS through NADPH oxidase as indicated by partial inhibition with apocynin. Li et al found that ET-1 produces ROS partially via NADPH oxidase in a rodent model [646]. Our results are also supported by Loomis et al who demonstrated in the rat aorta that ET-1 mediates free radical injury through eNOS uncoupling and NADPH oxidase [610].

The acute effects of ET-1 impairment on NO bioavailability and oxidative burst may explain poor outcomes acutely following coronary bypass surgery or cardiac transplantation [17]. Ozedemir et al reported in an MI model that ET-1 increases ROS with resultant myocardial injury and that ET-1 antagonism reduces ROS, improves antioxidant status and decreases myocardial injury [922]. Elevation of ET-1 early following transplantation results in endothelial dysfunction, allograft failure and an inability to wean from cardiopulmonary bypass [17]. Elevations in ET-1 have also been demonstrated to increase infarct size after
myocardial infarction. Prolonged exposure to ET-1 with the resultant impairment of NO production and increased production of ROS may lead to atherosclerosis, transplant coronary disease and pulmonary hypertension [33;563;564;600;607-609;976;978]. Several investigators have demonstrated that NADPH oxidase activation and eNOS uncoupling are implicated in endothelial dysfunction and atherosclerosis [979-983]. Hathaway et al found that vascular superoxide production is involved in the pathogenesis of atherosclerosis [980]. The importance of eNOS uncoupling was observed in the study by Alp et al where they showed that transgenic mice overexpressing GTP-cyclohydrolase (the enzyme responsible for BH₄ formation) when crossed with ApoE knockout reduced endothelial dysfunction and atherosclerosis [979]. Spiekermann et al as well as Warnholtz et al revealed that NADPH oxidase superoxide production is involved in endothelial dysfunction and atherosclerosis [982;983]. Superoxide production can impair NO bioavailability by forming PNT, by decreasing eNOS activity and uncoupling eNOS through BH₄ oxidation. Therefore, the ability of ET-1 to increase NADPH oxidase superoxide generation can further impair NO production beyond the direct effect of ET-1 on NO homeostasis.

6.4.2 EFFECTS OF PROTEIN KINASE ACTIVITY ON NO AND ROS PRODUCTION

6.4.2.1 ET-1 AND PROTEIN KINASE C

We found that NO production is regulated by PKC. We demonstrated that PKC inhibition resulted in a significant reduction in NO bioavailability while PKC activation led to NO release. The concomitant exposure to ET-1 and PKC inhibition did not lead to a synergistic decrease in NO bioavailability as would be anticipated if ET-1 acted solely in a PKC independent fashion. The simultaneous exposure of our endothelial cells to PMA and ET-1 increased NO production similar to exposure to PMA alone. Our data supports the
hypothesis that one pathway by which ET-1 decreases NO production is through PKC inhibition. eNOS protein expression was also regulated by PKC. We found that PKC antagonism downregulated eNOS protein expression while PKC agonists upregulated eNOS protein expression. ET-1 induced downregulation of eNOS protein expression was only seen following 24 hours of exposure indicating that NO impairment seen following 30 minutes of treatment is as a result of decreased eNOS activity and not through changes in protein expression. In addition, concomitant exposure of HSVEC to ET-1 and PMA resulted in upregulation of eNOS to a level similar to PMA alone further demonstrating that ET-1 induced alterations in eNOS expression is PKC dependent and is fully reversible by direct PKC stimulation. To determine which isoforms were involved in ET-1 regulation, we evaluated isoform specific translocation. Our study revealed that ET-1 exposure resulted in membrane translocation of the delta and epsilon isoforms while the M/C ratio of the lambda isoform was reduced suggesting inhibited translocation. We determined that cellular PKC activity is reduced following ET-1 exposure indicating that PKCλ is the likely isoform that regulates ET-1 effects on NO production. Since translocation does not always correlate with activity of atypical isoforms we measured PKCλ activity in our cell cultures. We confirmed that ET-1 exposure reduces PKCλ activity and by using the PKCλ inhibitor ZI we demonstrated that lambda inhibition impairs NO production. Using our results from the PKC translocation data we assessed whether PKCδ and PKCε play important roles in ET-1 induced changes in NO production. We found that PKCδ inhibition attenuated ET-1 impairment of NO homeostasis and did not reduce NO levels in control cells. Our findings indicate that PKCδ translocation and activation above baseline results in alteration of NO production since delta inhibition in control cells does not alter NO release. ET-1 mediated
translocation of PKCδ leading to impaired NO release may be as a result of a shift in the eNOS state from an NO producer to a ROS generator. This hypothesis is confirmed by our ROS data demonstrating that PKCδ inhibition reduces ET-1 induced ROS production. Therefore, PKCδ activation by ET-1 results in impaired NO production by altering the eNOS functional state leading to ROS generation over NO production. PKCδ inhibition partially attenuated ET-1 induced effects indicating that other isoforms and/or protein kinases are involved in NO production. Examination of PKCε inhibition revealed a significant reduction in both NO and ROS release in control and ET-1 exposed cells. The ability of PKCε inhibition to simultaneously lower NO and ROS levels indicates a reduction in eNOS activity. Therefore, PKCε translocation and activation results in eNOS activation and depending on the functional state of eNOS can lead to NO or ROS production. Kwan et al and Xia et al demonstrated that PKC can play a role in ROS generation [432;435]. They showed in mesangial cells that PKCζ is involved in ROS generation [432;435]. Their study also reveals that atypical PKC isozymes can regulate ROS formation [432;435]. ET-1 appears to have three effects on PKC activity in our isolated HSVECs model: it reduces lambda activity and activates both delta and epsilon leading to decreased NO production through decreased eNOS activity (PKCλ) and increased ROS production (PKCδ and PKCε). The differential roles of PKC on NO production have been observed in various animal models [428;430;436]. Matsubara et al in bovine aortic cells found that PKC activation decreases NO production while Partovian et al found that PKC activation increases NO formation [428;430]. Partovian et al found that PKCα activation increases eNOS activity in the vasculature indicating that PKC regulation of eNOS is tissue and species dependent [430]. Naruse et al demonstrated in the rat vasculature that PKCβ
activation decreases NO production however, PKCβ inhibition was mediated by Akt/PKB [436]. Their data supports our hypothesis that human endothelial cell may reduce NO by Akt/PKB inhibition [436]. Our studies found that PKCλ translocation was inhibited by Akt/PKB indicating that ET-1 may reduce NO production by decreasing Akt/PKB which decreases PKCλ activity leading to reduced NO production. However, we have demonstrated that Akt/PKB can also be inhibited by PKC modulation revealing that ET-1 induced PKC changes alters Akt/PKB activity and decreases NO production. Therefore, ET-1 decreases NO production by modulation of both PKC and PKB activity which then directly alters NO and ROS production. In addition, PKC alters Akt/PKB activity while Akt/PKB can modify PKC activity feeding the cycle and further impairing NO production. Broad inhibition or activation of PKC resulted in reduced serine phosphorylation of Akt/PKB. PMA, a non-specific PKC activator, may result in several changes in intracellular signaling that ultimately leads to a paradoxical inhibition of Akt/PKB. This effect is not seen with threonine phosphorylation. The effects of PKC modulation, on regulation of Akt activity, are far more complex than previously thought and may explain why broad PKC inhibition or activation have similar effects.

ET-1 induced alterations in NO homeostasis can also be a result of increased caveolin-1 expression leading to greater eNOS-caveolin-1 interaction and inhibition of eNOS. ET-1 and protein kinase modulation (PKC, PKB, PKA) did not affect caveolin-1 expression. NO production can also be regulated by increasing eNOS-caveolin-1 binding. Our investigations demonstrated that ET-1 exposure increases eNOS expression on the membrane fraction. The higher eNOS M/C ratio with ET-1 evident after both 30 minutes and 24 hours of exposure suggests inhibition of eNOS as sequestered eNOS is less active or inactive. The acute effect
of ET-1 on eNOS cellular location explains the decreased NO production observed following 30 minutes of exposure and further confirms the conclusion that ET-1 decreases eNOS activity. PKC inhibition resulted in a higher M/C ratio compared to control while PMA resulted in a reduced M/C ratio. Therefore, PKC can regulate eNOS localization. Another mechanism by which ET-1 reduces NO production is through PKC inhibition resulting in eNOS sequestration. ET-1 leads to a greater eNOS M/C ratio than PKC inhibition alone signifying that ET-1 sequesters eNOS by an additional pathway. The ability of ET-1 to increase caveolin-1 interaction has been previously demonstrated in mesangial cell were ET-1 increased ERK1/2 interaction with caveolin-1 [984]. We also assessed whether ET-1 results in iNOS induction. However, neither ET-1 nor PKC modulation induced iNOS. In summary, our results suggests that ET-1 modulates PKC translocation and activity which alters NO and ROS homeostasis.

6.4.2.2 ET-1 AND PROTEIN KINASE B

Akt/PKB has a significant role in NO homeostasis. Akt/PKB inhibition with SH5 reduces NO production in control cells demonstrating the importance of Akt/PKB in basal NO production. We observed an additive effect when ET-1 and SH5 were co-incubated therefore confirming that ET-1 does not solely act through Akt/PKB. ET-1 induced changes in PKC activity play a critical role in NO homeostasis. We ascertained that ET-1 related impairment in NO production was also driven by Akt/PKB. ET-1 exposed cells demonstrated reduced Akt/PKB activity. Therefore, ET-1 also impairs NO homeostasis by inhibiting Akt/PKB activity. Several studies support our conclusion that Akt/PKB regulates NO production [301;562;985;986]. Our novel finding is that elevated levels of ET-1 inhibit NO production via Akt/PKB inhibition. Akt/PKB can directly signal eNOS [301;562]. Our
data demonstrates that Akt/PKB can directly and indirectly through PKCλ alter eNOS driven NO production. Michell et al also showed that Akt/PKB can directly activate eNOS [562]. In our study SH5 decreases PKCλ translocation and PKC activity which can lead to decreased NO release. We have demonstrated that PKC modulation partially (but not fully) alters ET-1 induced ROS release indicating that multiple mechanisms are involved in ET-1 induced ROS production. SH5 mediated Akt/PKB inhibition resulted in increased ROS production. ET-1 which reduced Akt/PKB activity also led to increased ROS. Greater production of ROS is seen when SH5 is co-incubated with ET-1 due to the effects of ET-1 mediated PKC alterations in addition of Akt/PKB inhibition. We found that Akt/PKB inhibition did not alter eNOS, iNOS and caveolin-1 expression. Akt/PKB inhibition results in eNOS membrane localization demonstrating the role of Akt/PKB in ET-1 induced eNOS sequestration. In summary, our results suggest that ET-1 inhibits Akt/PKB resulting in eNOS membrane sequestration, reduced NO production and increased ROS formation.

6.4.2.3 ET-1 AND PROTEIN KINASE A

ET-1 did not alter PKA activity compared to control. We did not find that PKA modulation altered NO or ROS production in our endothelial cells nor did it alter PKC activity. A recent study by Hashimoto et al demonstrated that cilostazol, an inhibitor of phosphodiesterase III, exposure can increase NO production through a PKA dependent pathway [504]. However, they found that cilostazol also alters Akt/PKB activation which is known to result in NO production. Our data indicates that PKA does not play a role in baseline NO production nor in ET-1 induced NO impairment. One possible explanation for the findings of Hashimoto et al is that PKA activation led to Akt activation resulting in NO production. However, we did not find in our experimental protocols that PKA activation led
to NO production. ROS production was not altered by PKA activity. PKA modulation did not alter eNOS, iNOS, caveolin-1 expression or eNOS sequestration. No effect on PKC translocation or activity was seen following FOS or H89 exposure. Therefore, our studies suggest that ET-1 does not modulate NO homeostasis through a PKA dependent pathway.

6.4.3 EFFECTS OF ET-1 ANTAGONISM WITH BOSENTAN

We found that 24 hour exposure to ET-1 led to decreased eNOS protein expression. Dong et al. also demonstrated that elevated ET-1 results in downregulation of eNOS protein expression in endothelial cells and Wedgwood et al demonstrated that high ET-1 concentrations reduce NOS activity and expression in SMCs [607;609]. The inhibition of eNOS expression was abrogated by BOS treatment and/or PKC stimulation with PMA. Cellular localization of eNOS following ET-1 exposure appears to be shifted towards the membrane while BOS treatment appears to preserve eNOS in its more active cytoplasmic form. Since caveolin-1 expression is unchanged by ET-1, eNOS translocation is likely due to increased binding to caveolin-1.

Our study also sought to determine whether ET-1 blockade with BOS prevented ET-1 induced endothelial injury. Assessments of NO production demonstrated that BOS had the ability to increase basal NO release in addition to abrogating ET-1 induced effects. This was seen both acutely and with prolonged exposure. BOS attenuated ET-1 induced downregulation of eNOS expression while maintaining an M/C ratio similar to control. BOS also attenuated ET-1 generated ROS production. The ability of BOS to decrease ROS production even compared to control is likely unrelated to its ability to block baseline ET-1 levels. Since BOS is able to increase NO production, the increased NO likely quenches the ROS which is cleared by the cell. Furthermore, BOS treatment inhibited ET-1 induced
translocation of PKCδ and PKCε. PKCλ demonstrated greater translocation to the membrane following BOS exposure compared to ET-1 and control. PKC activity was increased at both 30 minutes and 24 hours following BOS exposure. BOS demonstrated the simultaneous ability to attenuate ET-1 induced endothelial dysfunction and improve NO homeostasis. These effects of BOS seem to further support the position that PKCλ is the key isoform involved in NO regulation. In support of this conclusion 1) PKC inhibition decreased NO production 2) PKC activation increased NO production 3) the presence of BOS results in increased NO production associated with increasing PKCλ translocation and overall PKC activity 3) ET-1 increased cytosolic expression of PKCλ while decreasing overall PKC activity 4) PKC activation with PMA translocated PKCλ while inhibition with CALC and Chelerythrine increased cytosolic expression in the control group.

The beneficial effects of ET-1 antagonism have previously been observed following I/R, in pulmonary hypertension and in transplantation [17;19;601;934]. Gonon et al demonstrated that NO mediates the myocardial protective effects of BOS during I/R [19]. Our data indicates that BOS’s protective effects also extend to the endothelium. Barton et al also showed that ET-1 blockade restores NO-mediated endothelial function in atherosclerotic mice [601]. We have previously demonstrated that BOS enhances both myocardial and endothelial protection during cardiac allograft storage [17]. The present study demonstrates the mechanism behind ET-1 injury and the putative beneficial effects of BOS treatment.

In summary, prolonged treatment of HSVECs to elevated levels of ET-1 decreases NO bioavailability through the modulation of PKC translocation and activity and Akt/PKB activity. HSVEC exposure to ET-1 decreased PKCλ activity and eNOS expression while
increasing PKCδ, PKCε activity and ROS production. ET-1 reduces Akt/PKB phosphorylation leading to impaired NO production, increased ROS formation and eNOS sequestration. ET-1 inhibition of Akt/PKB also decreases PKCλ activity and therefore ET-1 through Akt/PKB directly and indirectly (via PKCλ) alters NO homeostasis. ET-1 antagonism with BOS restored the NO-ET-1 balance. ET-1 antagonism may prove to be a novel therapeutic strategy to improve vascular homeostasis following I/R, heart failure and cardiac transplantation. We have previously demonstrated the beneficial effects of BOS following I/R [17]. Our study demonstrates that this benefit may be as a result of improved vessel homeostasis and that PKC and PKB play a critical role in this balance. Targeted PKC and/or PKB modulation may prove to be a superior strategy as it has the potential to augment the beneficial effects of ET-1 antagonism. Improved endothelial protection against ET-1 induced dysfunction offers the potential of reducing the negative effects of heart failure, decreasing the development of atherosclerosis and reducing allograft failure and CAV.

6.5 THE INTERACTIVE EFFECTS OF HYPOXIA/REPERFUSION AND ENDOTHELIN-1 ON ENDOTHELIAL FUNCTION, VIABILITY AND SURVIVAL: THE ROLE OF PROTEIN KINASES

Cardiovascular diseases account for a significant burden of disease in the Western World. CAD, MI, and heart failure are the principal causes of death. Those with end-stage heart failure or inoperable CAD have heart transplantation as a last resort for increasing life-expectancy and improving quality of life. The results of cardiac transplantation are limited by early and late mortality [34]. Early mortality is principally caused by primary allograft dysfunction/failure whereas late mortality is caused by CAV [34]. Primary allograft failure has several causes however, I/R injury is the main determinate of allograft failure. CAV
etiology is multifactorial and includes I/R injury, NO impairment, ET-1 and immunosuppressive drug injury. I/R injury is therefore an important determinate of outcomes following cardiac transplantation by limiting early and late survival. NO impairment seen in the cardiovascular patient is due to several factors such as atherosclerosis, CMV infection, I/R, hypertension, hyperlipidemia and alterations in vasoactive peptides such as ET-1. ET-1 levels are elevated following MI, in heart failure patients and in cardiac transplant recipients.

I/R depletes ATP stores and decreases intracellular pH. The production of free radical increases with deleterious consequences including protein and membrane damage. Other important negative aspects of I/R include impaired NO homeostasis and increased ET-1 production. Alter NO homeostasis is a hallmark of endothelial dysfunction. NO deficiency results in poor vasomotor function, increased endothelial cell activation and increased vasomotor sensitivity to vasospasm [12;126-130]. As we demonstrated in chapter three, elevated ET-1 levels are associated with decreased NO production, increased ROS formation and endothelial cell activation. ET-1 also alters protein kinase activity such as PKC and Akt/PKB. I/R is known to alter protein kinase (PKC and Akt/PKB) activity in several cell types.

Methods to reduce the injury from I/R would offer significant benefit to the transplant recipient. We have previously demonstrated that continuous or intermittent perfusion of the cardiac allograft reduces dysfunction and increases survival [17;99;100;936]. These investigations showed that H/R offers superior protection than cold ischemia reperfusion. This protection was seen in the myocardium as well as the endothelium. We have also found that ET-1 antagonism and NO augmentation during
hypoxia further enhanced protection [17;936]. The mechanisms of injury during H/R as well as the protective effects of BOS are unknown in human endothelial cell.

We hypothesized that H/R results in endothelial dysfunction and cell death marked by impaired NO production, increased ROS formation, altered protein kinase activity and induction of caspase activity. We proposed that ET-1 aggravates H/R injury and that BOS protects the endothelial cell form both H/R and ET-1 induced effects. We also investigated whether BOS has PC like properties by reducing reperfusion injury when given during hypoxia alone. Given the importance of maintaining endothelial function following H/R we sought to determine the mechanisms by which H/R and high levels of ET-1 impair the endothelium and result in cell death.

Our study revealed the following observations:

1. H/R and ET-1 alter PKC and Akt/PKB activity with no effect on PKA activity.
2. ET-1 exposure heightens H/R cell death.
3. H/R and ET-1 induces apoptosis through caspase 8 and caspase 9 activation.
4. PKC and Akt/PKB modulate cell death by regulating apoptosis.
5. ET-1 increases H/R induced impairment of NO production and ROS formation.
6. ET-1 derives ROS from eNOS uncoupling and NADPH oxidase activation.
7. NO impairment is accentuated by ET-1 exposure during hypoxia.
8. PKC as well as Akt/PKB play important roles in NO homeostasis under hypoxic conditions
9. H/R increases eNOS M/C ratio.
10. BOS treatment during hypoxia abrogates the effects of H/R and ET-1 during both the hypoxic phase and reperfusion phase.
11. ET-1 treatment during reperfusion worsens endothelial injury and death.

12. BOS treatment during reperfusion offers further protection.

13. PKCδ and PKCε inhibition protects against apoptosis while PKCλ inhibition increases apoptosis.

6.5.1 EFFECT OF H/R ON PROTEIN KINASE ACTIVITY

6.5.1.1 Effect of H/R on PKC Activity and Translocation

We demonstrated in chapter three that protein kinases play important roles in NO and ROS production. Several investigators have demonstrated that I/R as well as H/R can alter protein kinase activity in various cell types [420;421;987-990].

H/R increased consensus PKC activity compared to normoxia. Goldberg et al demonstrated that in myocytes H/R results in PKC activation [989]. Conte et al observed in a rodent model of colonic H/R increased PKC activity in colonic mucosa [988]. Exposure to ET-1 during hypoxia reduced PKC activity compared to hypoxia alone but PKC activity was still higher than that of normoxic exposure to ET-1. In addition, the inhibitory effect of ET-1 on consensus PKC activity was lost during reperfusion. BOS treatment reduced PKC activity during hypoxia and reperfusion. The similar action of ET-1 and BOS on consensus PKC activity during hypoxia is likely due to different effects on various PKC isoforms. BOS inhibits the effect of H/R on both delta and epsilon translocation which is likely the cause of the decreased consensus PKC activity even though PKCλ translocation is enhanced with BOS. In addition, BOS offers endothelial protective effects independent of its antagonism of ET-1 which may account for the reduction in consensus PKC activity. The effects of ET-1 on PKCλ activity may account for the reduction in consensus activity. Furthermore, ET-1 worsens endothelial cell injury which may account for indirect changes in PKC activity.
Differential effects of H/R on PKC have been observed in other cell types [988;989;991;992]. I/R also demonstrates a similar increase in PKC activity as well as a differential response of PKC isoforms [166-168;407;420;421;987]. The ability of ET-1 to exacerbate H/R injury was also demonstrated by Iwamoto et al in renal epithelial cells [990]. The ability of BOS treatment during hypoxia to decrease PKC activity during reperfusion demonstrates PC like effects. The role of PKC in PC has been previously demonstrated in cardiomyocytes [183;416;419;993-997]. Ping et al demonstrated that PKCε and PKCη activation leads to cardioprotection in rabbit cardiomyocytes while Liu et al found that PKCε (but not PKCδ) activation is involved in rodent myocardial PC [994;996]. In hepatocytes, Carini et al observed that PKCδ is a player in preconditioning [517]. Inagaki et al found that PKCε activation and PKCδ inhibition leads to cardioprotection in a rodent model [166]. The role of PKC in PC is therefore, cell and species specific. Therefore, BOS treatment during hypoxia may lead to endothelial protection during reperfusion by it ability to alters specific isoforms of PKC.

Analysis of isoform specific translocation was performed to further determine the role of PKC in H/R. H/R induced significant changes in PKC translocation and activity. The novel isoforms PKCδ and PKCε were significantly translocated to the membrane after hypoxia with a reduction in PKCλ translocation. After one hour of reperfusion, PKCδ translocation increased with a redistribution following 24 hours of reperfusion, although levels remained greater than normoxia. Churchill et al also reported in an isolated rat heart model of I/R that PKCδ translocates during reperfusion [998]. PKCε translocation during reperfusion gradually decreased back to normoxic levels after 24 hours of reperfusion. PKCλ revealed increasing membrane localization towards near normoxic levels at 24 hours.
of reperfusion. The observation that hypoxia differentially effects PKC isozymes has been demonstrated in previous studies [166;420;421;989;998]. In neonatal cardiomyocytes, Goldberg et al found that PKC\(\varepsilon\) and PKC\(\alpha\) were translocated to the membrane while PKC\(\delta\) was inhibited and no change in PKC\(\lambda\) translocation was observed [989]. In mature cardiomyocytes, Inagaki et al demonstrated that PKC\(\varepsilon\) inhibition and PKC\(\delta\) activation leads to ischemic injury [166]. Our investigations revealed that PKC delta and epsilon activation as well as PKC\(\lambda\) inhibition leads to cell death in human endothelial cells. The role of ET-1 in H/R was also assessed. The use of BOS during hypoxia attenuated the effects of H/R on PKC\(\delta\) and PKC\(\varepsilon\) translocation. BOS exposure also demonstrated complete abrogation of PKC\(\lambda\) inhibition during H/R. Therefore, ET-1 plays a role in H/R induced changes in PKC translocation however, since BOS failed to completely restore the cellular localizations of PKC isoforms to normoxic distributions, H/R must alter PKC translocation by additional mechanisms. Previous investigators have demonstrated that I/R as well as H/R increase ET-1 levels [860;867;872;875;876;914]. Hu et al and Yamashita et al demonstrated that hypoxia induces ET-1 production via HIF-1 expression and recruitment of transcriptions factors that bind to the ET-1 hypoxia response element [860;867]. When ET-1 was given during hypoxia it accentuated hypoxia induced alterations in PKC translocation. We have demonstrated in chapter three that ET-1 can modulate PKC translocation and activity. In our hypoxic experiments we confirmed that ET-1 can also alter PKC following hypoxia and reperfusion. Jones et al observed that ET-1 heightened the myocardial PKC response to ischemia which was associated with greater myocardial injury [999]. We found that PKC delta and epsilon translocation to the membrane was further increased by ET-1 compared to hypoxia. ET-1 exposure during hypoxia increased PKC\(\delta\) and PKC\(\varepsilon\) translocation compared
to 30 minutes of normoxic ET-1 exposure. This data further supports the finding that hypoxia alters PKC translocation independently from ET-1. ET-1 treatment during hypoxia reduced PKC\(\lambda\) translocation compared to hypoxia alone and 30 minutes of normoxic ET-1 exposure. ET-1 therapy during hypoxia also had persistent effects during reperfusion as assessed by continued elevated PKC\(\delta\) translocation and reduced PKC\(\lambda\) translocation at one hour of reperfusion. Therefore, hypoxic production of ET-1 also affects PKC translocation during reperfusion.

6.5.1.2 Effect of H/R on PKA Activity

Neither H/R nor ET-1 treatment affected PKA activity. The ability of our assay to detect PKA activity was confirmed by the assessment of decreased PKA activity with H89 exposure and increased activity with FOS treatment. PKA modulation did not alter the effects of H/R on the parameters assessed in this study. Chen et al also found that ET-1 and ischemia mediate their effects through PKC and not via PKA. They demonstrated that ET-1 regulates monocyte chemoattractant protein-1 production in human brain endothelial cell through PKC and not PKA [914].

6.5.1.3 Effect of H/R on PKB Phosphorylation

Akt/PKB is an important player in cell survival and apoptosis with Akt/PKB inhibition leading to decreased anti-apoptotic activity [544-561]. Exposure to hypoxia reduces Akt/PKB phosphorylation compared to normoxia. Reperfusion results in a time dependent increase in Akt/PKB serine phosphorylation to levels that remain less than normoxia while threonine phosphorylation returned to normoxic levels after 24 hours of reperfusion. ET-1 appears to play a role in H/R induced inhibition of Akt/PKB phosphorylation. We found that ET-1 reduced Akt/PKB activation during normoxia and
following H/R and that BOS was able to attenuate Akt/PKB inhibition. BOS treatment did not fully restore Akt/PKB phosphorylation indicating that H/R reduces Akt/PKB activation by additional mechanisms other than ET-1. One mechanism by which hypoxia and reperfusion can alter Akt/PKB phosphorylation is through changes in PKC activity. We observed that PKC inhibition reduced both serine and threonine phosphorylation following H/R indicating that H/R and ET-1 can reduce Akt/PKB activity through inhibition of PKCλ. Since co-incubation of ET-1 with Cal or Chel further inhibits phosphorylation without further decreasing PKCλ translocation, it appears that ET-1 also inhibits Akt/PKB phosphorylation independently from PKCλ. Additional support for the ability of H/R and ET-1 to inhibit Akt/PKB independently of PKC is that exposure to the PKC agonist, PMA, does not fully counteract the effects of H/R and ET-1. We also observed that PMA exposure alone also reduces Akt/PKB phosphorylation during normoxia indicating that PKCδ and/or PKCε activation may lead to Akt/PKB inhibition. Therefore, Akt/PKB is inhibited by board inhibition of PKC and by non-specific activation of PKC. Our findings suggest that PKCλ inhibition and PKCδ and PKCε activation results in Akt/PKB inhibition. Other studies support our findings. Bright et al found that PKCδ leads to ischemic cell death by inhibiting Akt phosphorylation in a cerebral ischemia reperfusion model [420]. In cardiac I/R, Murriel et al showed that PKCδ activation inhibits Akt/PKB [1000]. PKC and PKB may interact with each other as demonstrated by the fact that Akt/PKB inhibition can inhibit PKCλ translocation and vice versa. Therefore, H/R and ET-1 leads to cell death by inhibition of Akt/PKB. Specifically, H/R and ET-1 causes PKCλ inhibition while activating PKCδ and PKCε resulting in Akt/PKB inhibition which leads cell death by caspase activation. In addition, ET-1 exposure can inhibit Akt/PKB independently of PKC leading to cell death.
6.5.2 EFFECT OF H/R ON NO HOMEOSTASIS

Several studies have demonstrated that NO production is decreased following I/R [12;126-130]. Engelman et al observed a reduction in NO release from coronary effluent following myocardial I/R [126]. We observed that hypoxic exposure impaired NO homeostasis compared to normoxia with no recovery seen during reperfusion. NO impairment is associated with several deleterious outcomes such as impaired vasodilation, increased neutrophil interaction and cell death [12;242;1001-1003]. Jones et al demonstrated NO impairment accentuates myocardial I/R injury [242]. They showed that eNOS knockout mice have greater injury than wild type mice [242]. Their data suggests that production of NO is crucial in preventing ischemic injury even though eNOS produces ROS which is associated with its own deleterious effects. Therefore, limiting ROS injury at the expense of NO production can worsen cellular injury and death. The presence of ET-1 during hypoxia further impaired NO production compared to both hypoxic and normoxic controls. Continued exposure to ET-1 during reperfusion further reduced NO release. As with ischemia, Bodi et al found that endothelial cells exposed to a hypoxic insult release ET-1 [1004]. Our data suggests that ET-1 plays a role in H/R mediated NO impairment as demonstrated by significant improvement in NO production with BOS treatment during hypoxia. In addition, ET-1 impairs NO production during reperfusion as indicated by improved NO homeostasis when BOS was given during reperfusion. Jernigan et al showed that ROS as well as ET-1 impaired NO induced pulmonary vasodilation after hypoxia [1005]. Continued therapy with BOS during H/R demonstrated superior protection. Gonon et al found that the protective effects of BOS against myocardial I/R was NO dependent [19]. Our investigations reveal that BOS in fact increases NO production compared to
hypoxic control following H/R. BOS treatment during hypoxia demonstrated an interesting effect in that NO production during reperfusion is improved instead of inhibited as observed in the hypoxic control group. BOS can therefore improve NO production during reperfusion even though treatment was given during hypoxia revealing PC like properties. Several investigators have shown that NO may be a key player in PC [1006-1010]. This effect may be mediated by BOS’s ability to alter PKC activity in addition to its ET-1 antagonistic function. In further support of this hypothesis, endothelial cells exposed to PMA during hypoxia also demonstrated improved NO production during reperfusion. Although, BOS improved NO homeostasis it failed to restore NO production to normoxic levels, indicating that NO impairment during hypoxia is also affected by mechanisms other than increased ET-1 production.

PKC signaling is involved in NO homeostasis. We found that PKC inhibition following hypoxia significantly reduces NO production while PMA increased NO production. Even though PMA improves post hypoxic NO homeostasis, it does not restore NO production to levels seen with PMA exposure during normoxia. This finding indicates that H/R reduces NO production by additional means other than PKC modulation. Isoform specific inhibition determined the role of PKCδ, PKCε and PKCλ in NO homeostasis following H/R. H/R impairs NO production during hypoxia by reducing PKCλ translocation/activity and impairs NO production during reperfusion due to increased PKCδ and lower PKCλ activity. ET-1 aggravates H/R induced endothelial injury by further increasing PKCδ translocation/activity and decreasing PKCλ translocation/activity. PKCε inhibition during H/R further decreased NO production demonstrating that H/R and ET-1 mediated translocation/activity of PKCε results in increased NO production and counteracts
some of the effects of PKCδ, PKCλ and PKB inhibition. However, PKCε translocation/activity also resulted in increased ROS production. Therefore, the improved NO homeostasis may come at a price of increased ROS production. Omiyi et al and Wang et al showed that PKC is involved in myocardial ischemic injury [1011;1012]. Omiyi’s data supports the conclusion that PKCβ activation leads to decreased NO production while Wang’s results indicate that PKC activation leads to NO production [1011;1012]. Their studies confirm our findings that specific isoform modulation alters NO production following H/R. We also found that H/R reduces Akt/PKB phosphorylation leading to deceased NO formation. SH5 exposure during hypoxia further lowers NO formation compared to hypoxic controls and SH5 exposure during normoxia, demonstrating the ability of Akt/PKB inhibition to reduce NO production during H/R. H/R decreases Akt/PKB partially though ET-1 as BOS exposure improved Akt/PKB phosphorylation and NO production. Co-incubation with ET-1 further reduced NO production following H/R indicating that ET-1 either further reduces Akt/PKB activity and/or acts via another signaling pathway such as PKC and PKA. We have shown that ET-1 does in fact alter both PKC and Akt/PKB activity compared to H/R. Therefore, H/R and ET-1 exposure impairs NO production by increasing PKCδ translocation/activity and reducing PKCλ and Akt/PKB activity. PKA did not play a role in H/R or ET-1 induced alterations in NO homeostasis.

NO production can be altered by change in eNOS protein expression or activity. We observed that H/R significantly reduces eNOS expression following hypoxia with a gradual increase in expression during reperfusion back to normoxic levels after 24 hours. The ability of I/R to decrease eNOS expression followed by a gradual rise in expression have been demonstrated in neural and microvascular endothelial cells [1013;1014]. H/R seems to
possess the same pattern of eNOS expression. This increased eNOS protein expression may help maintain NO production; however, this may be associated with the negative consequence of increased ROS formation. We speculate that eNOS upregulation during reperfusion may be detrimental as it is not associated with improved NO homeostasis in the control group suggesting increased ROS production. We have observed that H/R results in impaired NO production while increasing ROS generation. Liu et al demonstrated that following cardiac ischemia reperfusion was associated with increased PNT due to increased NO and ROS from eNOS [150]. Several investigators have confirmed that PNT plays a role in cardiac I/R [150;153;1015;1016]. eNOS plays an important role as a source of free radicals during reperfusion. ET-1 further impairs eNOS protein expression following hypoxia compared to control. ET-1 is associated with NO impairment and greater cellular ROS production by either increasing eNOS uncoupling and/or NAPDH oxidase activity. We have demonstrated that ET-1 generates free radicals by both mechanisms. BOS treatment during hypoxia abrogated the H/R and ET-1 induced reduction in eNOS expression. The ability of BOS to maintain eNOS protein expression indicates the role of ET-1 in H/R induced impairment of eNOS expression. BOS improves NO homeostasis by maintaining eNOS expression while simultaneously preventing ROS formation. This avoids the deleterious effects seen in the control group whereby increasing eNOS expression during reperfusion led to higher ROS production. H/R decreases eNOS protein expression by inhibiting PKC activity which is supported by the observation that PKC inhibition decreases eNOS expression while PMA abrogates the effect of H/R. Although, PMA maintains eNOS expression and increases NO production it is associated with increased ROS formation and therefore endothelial injury and dysfunction. In addition, ET-1 induced changes in eNOS
expression are also mediated by PKC. Both H/R and ET-1 reduces PKC\(\lambda\) activity, suggesting that eNOS expression following hypoxia and reperfusion is mediated by PKC\(\lambda\). PKA and PKB did not alter H/R and ET-1 effects on eNOS expression.

Changes in eNOS localization also affect NO production. As eNOS localizes to the membrane, NO production decreases. H/R increases eNOS membrane localization with a higher M/C ratio seen with ET-1 exposure. The role of ET-1 in H/R eNOS localization is supported by the ability of BOS to preserve cytoplasmic eNOS. We observed a significant role of both PKC and PKB in eNOS localization following H/R while PKA modulation demonstrated no effect. We found that PKC and PKB inhibition increased eNOS membrane localization while PMA decreased the M/C ratio for eNOS. Therefore, H/R and ET-1 which reduced PKC\(\lambda\) and PKB activity leads to increased eNOS membrane sequestration and decreased NO production. eNOS localization is determined by binding to caveolin-1. This can be achieved by increased affinity of eNOS for caveolin-1 or increased caveolin-1 expression. We found no significant effect of any treatment group on caveolin-1 expression therefore, eNOS localization to the membrane is increased through heightened eNOS to caveolin-1 binding following H/R. Hua et al previously demonstrated that ET-1 can increases caveolin-1 binding to other membrane associated proteins [984].

In summary, H/R impairs NO production. H/R leads to PKC\(\lambda\) and PKB inhibition while activating PKC\(\delta\). These PK changes results in NO impairment by reducing eNOS expression, activity and by increasing eNOS membrane sequestration. H/R induced NO dysregulation is also caused by ET-1 as indicated by improved NO homeostasis by ET-1 antagonism. ET-1 then leads to PKC\(\lambda\) and PKB inhibition while activating PKC\(\delta\) resulting in decreased NO production. H/R regulates NO impairment by altering PK activity and ET-
1. In turn, ET-1 also alters PK activity resulting in further NO impairment. H/R reduces PKB activity by several mechanisms including increasing ET-1 production, PKCδ/ε activity and by PKCλ inhibition. Therefore, H/R and ET-1 alters NO homeostasis by inhibiting PKCλ and PKB while activating PKCδ.

**6.5.3 EFFECT OF H/R ON ROS HOMEOSTASIS**

It has been well established that I/R generates ROS [659;1005;1016-1019]. We demonstrated that hypoxia as well as reperfusion increased endothelial cell ROS production compared to normoxic exposure. ET-1 exposure during hypoxia enhanced free radical generation both after hypoxia and during reperfusion. ET-1 treatment during hypoxia and reperfusion further increased ROS production. ROS production was proportional to reperfusion time which is logical as longer reperfusion time provides more oxygen exposure and a greater opportunity to produce free radicals. We demonstrated in chapter four that ET-1 can produce ROS, which is consistent with the findings of other investigators. [609;910] Wedgwood et al found that ET-1 impairs eNOS expression by a ROS mediated process. [609] Therefore, ET-1 induced ROS production may further downregulate eNOS expression following H/R. BOS therapy during hypoxia protected against H/R and ET-1 induced free radical release, thus underscoring the role of ET-1 in hypoxia induced ROS production. Therefore, one mechanism by which H/R increases ROS is through ET-1 driven free radical formation. H/R induced ROS formation can also lead to decreased eNOS expression and lower NO production. The protective effects of BOS also extended to the reperfusion phase indicating a PC like effect of BOS on ROS formation. We speculate that the ability of BOS to increase NO production while reducing ROS formation confers protection against the oxidative burst seen during reperfusion. We also hypothesized that this protection will lead
to decreased cell death. Our caspase activity assessment demonstrated that BOS in fact has PC like properties. BOS therapy during reperfusion also limited reperfusion induced ROS production indicating the role of ET-1 in reperfusion induced free radical formation.

PKC played a significant role in H/R induced ROS production. PKC inhibition resulted in a reduction of ROS while activation with PMA increased ROS production following H/R. We have demonstrated that under normoxic conditions PKC regulates ROS production. Previous studies have revealed that PKC activation increases ROS while inhibition decreased ROS formation [1020-1022]. Churchill found that PKC activation leads to ROS formation following I/R [1023]. Kwan et al showed that atypical PKC isozymes can regulate the generation of ROS [435]. Co-incubation of ET-1 with Cal, Chel, and PMA resulted in increased ROS production compared to Cal, Chel and PMA alone. Therefore, H/R as well as ET-1 increases ROS by additional signal transduction pathways. PKA modulation did not affect H/R and ET-1 induced ROS release. Akt/PKB inhibition during hypoxia was found to increase free radical formation following both hypoxia and reperfusion. Therefore, ROS formation following H/R involves ET-1 release as well as PKC and PKB modulation.

Isoform specific PKC inhibition was performed to further determine the role of specific PKC isozymes involved in ROS generation. The inhibition of both PKCλ and PKCε exposure during hypoxia resulted in significant ROS reduction after hypoxia and reperfusion with additional treatment during reperfusion resulting in a further reduction. PKCδ inhibition lowered free radical formation only when given during reperfusion. The ability of PKCδ to increase ROS production during reperfusion was also observed by Churchill et al [1023]. In the ET-1 exposed group all isoform specific inhibitors decreased ROS production
when therapy was during hypoxia and increased benefit was observed with treatment during reperfusion. Our data indicates that hypoxia produces ROS by increasing PKCε activity while reperfusion results in increased PKCδ activity and persistent ROS generation. Bouwman et al suggested that ROS can increase PKCδ activation which may explain our finding that PKCδ translocation is increased during reperfusion as ROS production is heightened [1024]. In the presence of elevated levels of ET-1, PKC isozyme activity is further altered leading to increased ROS formation.

We defined the signal transduction pathways, but the sources of free radicals induced by H/R and ET-1 remain unclear. Using specific antioxidant therapy we observed that eNOS and NADPH oxidase were the source of free radicals produced following H/R and by ET-1. The use of sepiapterin demonstrated that eNOS uncoupling was a mechanism of ROS formation. Landmesser et al reported that BH₄ can be oxidatively degraded and therefore H/R mediated ROS production can further uncouple eNOS and lead to more free radical formation [1025]. They also demonstrated that NADPH oxidase amplifies oxidative stress through increased eNOS driven ROS production by decreasing BH₄ levels.[1025] In addition, activation of NADPH oxidase is another mechanism by which H/R and ET-1 impairs NO production by uncoupling eNOS via ROS injury. NADPH oxidase is not the sole mechanism by which eNOS is uncoupled as inhibition of NADPH oxidase with apocynin does not fully restore oxidative stress back to normal. Manevich et al demonstrated that NADPH oxidase can be activated by I/R and in our study we confirmed that H/R can also activate NADPH oxidase [1026]. Gorlach et al observed that NADPH oxidase is activated by PMA in endothelial cells and we therefore speculated that PKCδ and/or PKCε were involved in NADPH oxidase activation.[1027] The observation that TE and TI provide
less protection against ROS generation than sepiapterin, L-NAME or apocynin suggests that H/R and ET-1 reduces SOD activity and/or overwhelms the ability of catalase or glutathione peroxidase to clear hydrogen peroxide. Alternatively, H/R and/or ET-1 may inhibit catalase and glutathione peroxidase activity directly. We did not investigate this possibility. iNOS can also be a source of ROS, but we found no significant induction of iNOS following either H/R or ET-1 treatment. Antioxidant protection against the generation of free radicals extended to the reperfusion phase even when treatment was confined to hypoxia. This reveals a PC like effect and may be a mechanism by which BOS confers protection against injury. The reduction in ROS formation by BOS may be another pathway by which BOS improves NO bioavailability, since limiting ROS generation will decrease BH$_4$ oxidation and increase eNOS dimerization leading to increased NO production. The supplementation of antioxidants during reperfusion further limited the development of ROS. We observed that eNOS and NAPDH oxidase are also involved in reperfusion induced free radical formation. Wang et al demonstrated PNT formation during reperfusion of the ischemic heart [1015]. Their data reveals that eNOS is a player in reperfusion induced ROS formation by producing both NO and superoxide.

In summary, hypoxia results in ROS formation by increasing PKC$_{\varepsilon}$ translocation and activity and by inhibiting PKB phosphorylation. Reperfusion increases ROS production through PKC$_{\varepsilon}$ and PKC$_{\delta}$ translocation and activation and by Akt/PKB inhibition. ET-1 release is another mechanism by which hypoxia and reperfusion generates ROS. ET-1 leads to PKC delta and epsilon translocation while inhibiting PKB. These changes are associated with increased ROS formation. Furthermore, exposure to elevated levels of ET-1 during H/R aggravates hypoxic and reperfusion induced ROS generation. H/R and ET-1 produces
ROS by shifting eNOS into a ROS generator instead of an NO producer and by NADPH activation. eNOS functional uncoupling is caused by PKCδ translocation and activation while increased eNOS activity and generation of ROS is do to PKCe translocation and activation.

6.5.4 H/R AND CELL VIABILITY

Rodriguez et al found that liver I/R decreased cell viability and energy stores [1028]. The ability of I/R to reduce cell viability in vascular endothelial cell was demonstrated by Serraf et al [1029]. We confirmed that human endothelial cells exposed to H/R had decreased cell viability. As expected, prolonged exposure to reperfusion further injures the endothelial cell as observed by a greater reduction in cell viability. The presence of ET-1 during hypoxia exacerbated the effects of H/R resulting in decreased cell viability. The importance of ET-1 in H/R injury was observed by the protective action of BOS. BOS treatment during hypoxia improved cell viability compared to control. The presence of BOS during reperfusion also improved cell viability and the continued therapy of BOS throughout hypoxia and reperfusion offered superior protection against the impairment in cell viability. This data indicates that ET-1 plays a role in both hypoxia and reperfusion induced reduction in cell viability. BOS also demonstrated PC like properties as the exposure to BOS during hypoxia limited endothelial injury during reperfusion. Our investigations indicate that H/R as well as ET-1 impairs endothelial function and survival.

We have shown that ET-1 alters PKC translocation and activity leading to endothelial dysfunction. PKC modulation also affected cell viability. Both inhibition and activation of PKC during hypoxia resulted in a significant reduction in cell viability. Our data indicates that cell viability is affected differently depending on the isoforms activated or
inhibited. PKC\(\lambda\) inhibition while PKC\(\delta\) and PKC\(\epsilon\) activation leads to decreased cell viability. Therefore, exposure to Cal and Chel results in impaired viability by inhibiting PKC\(\lambda\) and PMA reduces cell viability by increasing PKC\(\delta\) and PKC\(\epsilon\) activity. In addition, PKC modulation (activation/inhibition) also affects PKB, NO and ROS. Cal, Chel and PMA decreases Akt/PKB phosphorylation via different mechanisms but ultimately results in decreased cell viability. PMA exposure also generates ROS which decreases cell viability. Therefore, non-specific PKC modulation (activation/inhibition) through different mechanisms results in the same negative effect on cell viability. The co-incubation of Cal, Chel or PMA with ET-1 led to further impairment in cell viability indicating that ET-1 likely decreases cell viability by additional, non PKC dependent pathways. The exposure to PKA antagonists or agonists during hypoxia revealed no significant impact on either H/R or ET-1 induced impairment in viability. Therefore, H/R and ET-1 does not affect cell viability via alterations in PKA activity. We also assessed PKA activity following H/R and ET-1 exposure and found no significant activation or inhibition of PKA. Akt/PKB plays an important role in cell survival [561;1030-1032]. We hypothesized that H/R reduces cell viability by Akt/PKB inhibition. Assessment of Akt/PKC phosphorylation revealed that both H/R and ET-1 decreases Akt/PKB activity. SH5 treatment demonstrated the role of Akt/PKB in H/R. Akt/PKB inhibition with SH5 reduced cell viability following H/R. Since the co-incubation of SH5 and ET-1 further reduced cell viability, contribution from another pathway such as PKC must exist. BOS exposure during reperfusion also protected the endothelial cell injury following SH5 exposure during hypoxia indicating the protective effects of BOS against reperfusion injury regardless of the cause.
The use of zvad, a synthetic peptide that binds to the active site and inhibits activity of caspases, during hypoxia lowered the deleterious effect of prolonged reperfusion indicating that apoptosis plays a role in H/R impaired viability, but that inhibition of apoptosis during hypoxia alone is insufficient to limit the initiation of apoptosis during reperfusion. We speculated that the PC like effects of BOS may be due to altered apoptosis activation. The presence of zvad during both hypoxia and reperfusion prevented the reduction in cell viability seen with H/R. This result indicates that reperfusion injury is an important player in H/R induced apoptosis and endothelial cell injury. Even though viability is maintained with zvad treatment there was a significant impairment in cell function, growth and proliferation as demonstrated by the reduced cell viability compared to normoxic cells grown for 48 hours. We hypothesize that H/R impairs viability partially via mitochondrial dysfunction in addition to inducing cell death and limiting cellular growth and division. Mitochondrial dysfunction caused by cerebral I/R was reviewed by Fiskum et al [1033]. Churchill et al demonstrated that myocardial I/R can result in mitochondrial dysfunction [1023]. From our study results, we speculate that H/R through changes in ET-1 as well as PKC and PKB alter viability by impairing mitochondrial function, cell growth and inducing cell death (apoptosis).

6.5.5 H/R AND CELL DEATH

I/R has been well established to result in cell death in various cell types [1034-1044]. Cell death can be mediated by apoptotic or non-apoptotic pathways. I/R can activate both [1034-1044]. Our cell viability studies suggested that apoptosis was involved as zvad improved viability after H/R. Lee et al observed activation of caspase 3 leading to apoptosis in ischemic cerebral endothelial cells. [1045] They also confirmed the ability of zvad to
reduce caspase 3 activity [1045]. We found that H/R cell death was mediated through apoptosis as determined by caspase 3 activation. The assessment of non-apoptotic cell death showed that H/R cell death is not mediated by autophagy. No significant differences in necrosis were observed between groups following H/R. However, there was a trend towards increased necrosis with increasing reperfusion time. Our data indicates that necrosis is not a key player in H/R and ET-1 induced cell death. Induction of necrosis may be limited as the cells are hypoxic and not ischemic. The presence of a small amount of oxygen may prevent necrosis and favor an apoptotic cell death pathway. Furthermore, the reperfusion insult may be of a short duration and prolonged exposure may be required to observe greater necrosis.

Exposure to 24 hours of hypoxia led to a significant increase in apoptosis as assessed by caspase 3 activation with a further reperfusion time dependent increase in caspase activity. We speculate that hypoxia initiates apoptosis which is continued during reperfusion. In addition, reperfusion itself induces caspase activation and further promotes apoptosis. Gottlieb et al reported that reperfusion itself can cause apoptosis independently of ischemia [1035]. Vanden Hoek et al also demonstrated that reperfusion can lead to injury [1037]. Treatment with ET-1 during hypoxia further increased caspase 3 activation. The use of BOS during hypoxia revealed protection against the development of apoptosis during both hypoxia and reperfusion. This observation indicates that ET-1 plays a role in H/R induced apoptosis and that BOS can limit the effects of reperfusion even when given during hypoxia alone. Eigle et al demonstrated that ET-1 exposure can increase apoptosis in ischemic myocytes [1046]. Sekhon et al using a renal I/R model found that inhibition of ET-1 production limited apoptosis [1047]. In a lung transplant model Shaw et al observed that SB209670, an ET-1 antagonist, reduced apoptosis [1048]. Pear et al demonstrated in a
cardiac ischemia reperfusion model that BOS reduced myocardial apoptotic index as well as improved cardiac function [1049]. Pearl et al data indicated that reducing apoptosis can lead to improved myocardial recovery and function [1049]. We previously demonstrated that BOS can improve endothelial function following cardiac transplantation [17]. Treatment during reperfusion with BOS decreased caspase 3 activation following one and 24 hours of reperfusion; therefore, apoptosis occurs during both hypoxia and reperfusion. This observation also supports the hypothesis that ET-1 is involved in reperfusion induced cell death. The PC like effects of BOS are significant as the inhibitor zvad when given during hypoxia alone did not offer the same protection against apoptosis during reperfusion as did BOS. Peralta et al demonstrated in a hepatic ischemia reperfusion model that BOS pretreatment offered the same protection as IPC [1050]. Their results support our hypothesis that BOS treatment during hypoxia possesses PC like effects [1050]. The effect of BOS may extend beyond decreasing caspase activity, it may reduce the initiation of apoptosis or heighten the anti-apoptotic pathways.

The roles of protein kinases in endothelial cell H/R were assessed. We found that apoptosis involved both PKC and Akt/PKB but not PKA. The modulation of PKC activity (either activation or inhibition) showed a significant induction of caspase 3 activity. This result indicates that specific isoforms of PKC have different effects. Since both PKC activation and inhibition results in apoptosis we analyzed the response to isoform specific inhibition. We determined that PKCδ and PKCε inhibition protects against H/R induced apoptosis while PKCλ inhibition leads to cell death. ET-1 driven cell death was also attenuated by the use of PKC delta and epsilon antagonists. Therefore, H/R activates caspase 3 leading to apoptosis by inhibiting PKCλ. Caspase 3 is also activated by H/R through the
translocation and activation of PKCδ and PKCe. In addition, H/R alters isoform specific PKC changes in an ET-1 dependent and independent manner. H/R production of ET-1 leads to PKCλ inhibition, PKCδ and PKCe activation all of which results in caspase 3 activation and cell death. The interactive effect of elevated ET-1 levels with H/R further accentuates these PKC isoform specific changes leading to cell death by caspase 3 activation. Bright et al in a cerebral model of I/R demonstrated that PKCδ is a player in I/R and that inhibition of PKCδ reduces apoptosis [420]. Inagaki et al revealed that PKCδ inhibition protects the heart from reperfusion induced apoptosis [167]. Koponen et al observed that PKCζ inhibition reduced NMDA-induced cell death [1051]. Their data demonstrated that atypical PKC isoforms can play a role in cell death although their results demonstrated that activation leads to death [1051]. However, they used a different tissue and a different mechanism of cell death. Wang et al found that PKC inhibition can result in cell death [1052]. They showed that astrocyte cell death was enhanced when PKCβ is inhibited with no effect on cell survival by other isoform inhibitors while Omiyi et al found that PKCβ inhibition leads to cardioprotection against I/R [1011;1052]. Wang et al also observed that PKCe activation leads to protection in neural cells while PKCβ modulation had no effect on survival [1052]. Inagaki demonstrated in the myocardium that PKCe activation leads to protection from apoptosis [166]. Therefore, PKC’s effects on cell death are cell type and isoform specific. Our investigations reveal that H/R induced cell death is mediated by PKCδ and PKCe activation and PKCλ inhibition. BOS exposure limited apoptosis by preventing H/R induced alterations in PKC. The ability of BOS to limit apoptosis further supports the hypothesis that H/R mediates apoptosis partially via ET-1. Shizukuda et al demonstrated that PKCδ can regulate apoptosis via induction of ROS [423]. Their data suggests a role for ROS in the
modulation of signal transduction pathways leading to cell death. The co-incubation of Cal, Chel and PMA with ET-1 further increased apoptosis signifying that ET-1 acts through additional pathways than PKC. The assessment of Akt/PKB demonstrated that inhibition leads to apoptosis. H/R demonstrated a reduction is Akt/PKB phosphorylation and increased apoptosis. ET-1 exposure during hypoxia further decreased Akt/PKB phosphorylation while BOS exposure limited Akt/PKB inhibition. Bright et al showed that PKCδ inhibition increased Akt/PKB phosphorylation decreasing apoptosis [420]. Their data demonstrate that Akt/PKB is involved in apoptosis and that PKCδ may be upstream of Akt/PKB in a rodent hippocampal slice model of I/R [420]. Murriel et al showed that Akt/PKB dephosphorylation results in myocardial apoptosis [1000]. The ability of BOS to preserve Akt/PKB phosphorylation during hypoxia and increase activity during reperfusion is another mechanism for its protective effect and PC-like properties. The protective effects of increased Akt/PKB activity during reperfusion against apoptosis was demonstrated by several investigators [1053-1055]. An increased induction of apoptosis was observed when ET-1 was co-incubated with SH5 revealing that ET-1 acts through additional pathways than Akt/PKB alone. The ability of PKC to modify Akt/PKB activity and vice versa may lead to a cycle that synergistically increases cell death.

In addition to protein kinase modulation, ROS species can lead to apoptosis. Several studies have demonstrated that I/R results in ROS induced apoptosis [1056-1058], and we confirmed that H/R also increases ROS leading to the induction of apoptosis. In support of this statement we found that endothelial cells exposed to hydrogen peroxide had increased caspase 3 activation. Furthermore, the use of antioxidants decreased ROS production and apoptosis. Canatara et al showed that blocking caspase 3 activation limited hydrogen
peroxide induced apoptosis indicating that attenuating apoptosis may reduce ROS induced injury [1059]. We observed that antioxidant therapy during hypoxia offered protection against reperfusion induced cell death. This finding demonstrates the importance of free radicals in initiating apoptosis and a possible mechanism by which BOS confers PC like effects. BOS decreases ROS production during hypoxia which may account in part for its protective effects. We also revealed that continuing therapy with antioxidants during reperfusion further protects endothelial cells from cell death. Liu et al found that reducing intracellular ROS decreased HL-60 caspase 8 and 9 activation [1060]. Their data indicates that ROS can activate both the intrinsic and extrinsic apoptotic pathways. Antioxidant therapy demonstrated the source of ROS production. eNOS and NADPH oxidase were responsible for H/R and ET-1 driven ROS formation as inhibition with L-NAME and apocynin reduced free radical formation and apoptosis. Dodd et al revealed that apocynin can reduce ischemic lung injury and Wang et al observed that apocynin protected neuronal cells from death following I/R [656;1061]. In addition, eNOS uncoupling is one mechanism by which H/R induces ROS formation and cell death as treatment with sepiapterin (which is converted to BH₄) decreases ROS and apoptosis. Lin et al demonstrated that ROS such as PNT can induce apoptosis indicating a role for eNOS and eNOS uncoupling in ROS induced cell death [142]. Kidd et al (in cerebral tissue) as well as Schimid et al (in lung tissue) revealed that promoting eNOS dimerization reduces ROS production, cell injury and death [1062;1063]. ET-1 exposure further increases ROS production by increased NADPH oxidase activity and eNOS uncoupling resulting in cell death. TE and TI also provided protection against apoptosis however, to a lesser extent than the other antioxidants. This observation also supports the hypothesis that ROS results in apoptosis. Morita-Fujimura et
al demonstrated that overexpression of SOD can reduce apoptosis and more specifically caspase 8 activation [1064]. iNOS induction is another potential source of ROS. Ischemia and reperfusion have been demonstrated to result in iNOS induction leading to ROS formation and apoptosis however, following H/R we did not observe any iNOS induction [1065-1067]. Therefore, H/R does not produce free radicals or mediates apoptosis via iNOS. We have observed that both PKC and PKB alter ROS production therefore, PKC and PKB can directly and indirectly through ROS generation induce apoptosis. In addition, ROS can directly or indirectly (by altering PKC, PKB activity and by impairing NO production) result in cell death. Shizukuda et al did find that PKC can alter ROS and induce cell death [423].

Apoptosis can be initiated by either caspase 8 and/or caspase 9 dependent pathways. Kossmehl et al showed that both the intrinsic and extrinsic pathways are involved in cardiac I/R [1068]. We determined that H/R resulted in caspase 8 and 9 activation. BOS therapy during hypoxia limited caspase 8 and 9 activity during hypoxia and during reperfusion demonstrating PC like effects. Furthermore, this data indicates that ET-1 is a player in hypoxia induced caspase 8 and 9 activation. BOS given during reperfusion also reduced caspase activity demonstrating the role of ET-1 in reperfusion induced apoptosis. The exposure of ET-1 during hypoxia exacerbated apoptosis with reperfusion treatment further increasing caspase activity. PKC inhibition and activation increased caspase 8 and 9 activity confirming our caspase 3 assay result and indicating that specific PKC isoform changes have varied effects. Several investigators have demonstrated the ability of PKC to modulate caspase 8 and/or 9 activity [422;1069-1071]. Since ET-1 co-incubation further increase caspase 8 and 9 activity another signal transduction pathway must be involved. The inhibition of Akt/PKB is known to increase apoptosis. We observed that caspase 8 and 9
activity is increased following SH5 exposure with further activation when SH5 is co-incubated with ET-1. Herman et al found that Akt/PKB can alter caspase 9 activation while Okhrimen et al showed that Akt/PKB can modulate caspase 8 activity [422;561]. Skurk et al also demonstrated that Akt/PKB can modulate the extrinsic pathway (caspase 8) [559]. Therefore, caspase 8 and 9 are activated by H/R partially through ET-1 and by alterations in PKC and PKB activity. BOS prevented caspase 3, 8 and 9 activity which protected the endothelium from cell death. Sekhon et al demonstrated that triple protection with an antioxidant, an NO donor and an ECE inhibitor protected against renal I/R [1047]. Our data reveals that BOS results in NO production and decreased ROS generation; therefore, BOS has the ability to protect the endothelium by several mechanism with the use of one agent instead of three.

**6.5.6 SUMMARY**

In summary, as with I/R, H/R impairs endothelial function and induces cell death. Endothelial dysfunction was characterized by altered NO and ROS homeostasis. H/R regulated NO and ROS production by altering PKC and PKB activity. The modulation of PKC and PKB also alters cell viability and results in cell death by inducing both the intrinsic and extrinsic apoptotic pathways. We demonstrated that ET-1 is an important player in H/R induced changes in NO and ROS production and protein kinase activity. We defined that PKCδ and PKCe activation lead to apoptosis as does inhibition of the PKCλ isoform. This observation reveals that unlike in myocytes, PKCe activation is deleterious to the endothelial cell instead of protective [167;1072]. Another novel finding is that PKCλ inhibition is involved in cell death. Akt/PKB inhibition was found to be another pathway by which H/R results in cellular dysfunction and death. Our investigations showed that BOS treatment can
reduce the harmful impact from H/R. In addition, we observed that BOS can confer PC like properties when given during hypoxia which can be a valuable strategy of protection during allograft storage in order to reduce injury from transplantation. The protective effects of BOS likely come from its ability to improve NO homeostasis, reduce ROS generation and modify both PKC and PKB activity. We also found that ET-1 exposure exacerbates H/R injury indicating that patients with elevated ET-1 levels (such as those with heart failure) are more vulnerable to the effects of H/R. Therefore, those patients requiring a heart transplant are predisposed to heightened sensitivity toward H/R induced endothelial injury and allograft failure. We have previously demonstrated that BOS therapy can reduce primary allograft failure by improving both endothelial and myocardial function [17]. Yamaguchi et al showed that ET-1 antagonism can also reduce the development of CAV [103]. Our study provides that mechanisms behind endothelial injury which can lead to targeted therapies which can greatly enhance endothelial protection and reduce the development of both allograft failure and CAV, the principal determinates of outcome following cardiac transplantation.

We propose the following mechanisms by which H/R results in endothelial dysfunction and cell death. Hypoxic exposure translocates PKCδ and PKCε while increasing the cytosolic expression of PKCλ. PKB inhibition also occurs after hypoxia while no change in PKA activity was seen. Hypoxia induced inhibition of PKCλ results in NO impairment by decreasing eNOS expression and activity. ROS production during hypoxia results from hypoxic induced changes in PKCδ and PKCε translocation and activity. PKCδ and PKCε generate ROS production by uncoupling eNOS and activating NADPH oxidase. Hypoxic reduction in Akt/PKB activity generates ROS by inhibiting PKCλ, uncoupling
eNOS and by increasing NADPH oxidase activity. Furthermore, Akt/PKB inhibition also impairs NO formation during hypoxia by increasing the M/C ratio for eNOS and therefore reducing the production of NO. In addition, hypoxia activates caspase 3 and hence apoptosis via translocation and activation of PKCδ and PKCε as well as by PKCλ and Akt/PKB inhibition. Reperfusion also altered PK translocation and activity. NO impairment during reperfusion is caused by PKCδ reduction in NO production by uncoupling eNOS and by PKCε ability to generate ROS through eNOS and NADPH oxidase and by quenching NO. Akt/PKB also impairs NO production during reperfusion by altering the functional state of eNOS. Cell death following reperfusion is induced through inhibition of PKCλ and PKB and activation of PKCδ and PKCε. H/R also results in cell death by impairing NO production and by ROS generation. ET-1 exposure during hypoxia accentuates hypoxic injury. ET-1 accentuates hypoxic induced PK changes and further impairs NO production, increases ROS formation and cell death. PKCδ plays and important role in NO impairment in ET-1 exposed cell. ET-1 exposure during hypoxia leads to an additive increased in PKCδ translocation and activity resulting in eNOS uncoupling and a further decrease in NO production. Cell death is also increased by the additive effect of ET-1 on PKC and PKB activity. The enhanced cell death and NO impairment during reperfusion with ET-1 are also as a result of increased PKCδ and PKCε translocation and activity and further inhibition of PKCλ and PKB. We also demonstrated the protective effect of BOS during hypoxia and during reperfusion. BOS protective effects during hypoxia are principally driven by its ET-1 antagonistic action. BOS exposure normalizes PKB activity, PKCδ and PKCε translocation and activity limiting NO impairment, ROS production and cell death. In addition to blocking ET-1, BOS increases PKCλ translocation and activity and prevents PKB inhibition which
results in increased NO formation and protection against apoptosis by reducing caspase 3 activation. Furthermore, BOS has antioxidant properties which reduce ROS generation and cell death. The exposure to BOS also has preconditioning-like properties as BOS exposure during hypoxia alone protects against reperfusion injury. This protection is driven by BOS induced PKC and PKB changes which normalizes eNOS expression, activity and functional state while reducing ROS stress. BOS also protects against reperfusion injury by preventing PKCδ and PKCε translocation and activation preventing caspase 3 activation and eNOS derived ROS formation. In summary, H/R through the production of ET-1, changes in PKC and PKB activity lead to NO impairment, ROS production and cell death by altering eNOS expression, activity and functional state and caspase activation.

In these series of experiments, we observed the crucial role that ET-1 and protein kinase modulation plays in the development of endothelial dysfunction and cell death following H/R. We illustrated several strategies that can be utilized to minimize the deleterious effects of H/R. We demonstrated that ET-1 antagonism, PKC modulation as well as PKB activation can protect the endothelium from both hypoxic and reperfusion injury. Clinical studies are required to confirm the potential benefits of these strategies.

6.6 IMMUNOSUPPRESSIVE DRUG INDUCED VASOMOTOR DYSFUNCTION: THE ROLE OF NITRIC OXIDE AND ENDOTHELIN-1 REGULATION

6.6.1 INTRODUCTION

Our study confirms that CyA results in endothelial dysfunction. CyA treatment alters normal vascular homeostasis as demonstrated by impaired endothelial dependent vascular dilatation. Sudhir et al and Khalil et al showed in dogs that acute exposure to CyA impairs coronary endothelial function [719;1073]. Their results also indicate that NO dysregulation
may be involved in CyA-induced dysfunction [719;1073]. The novel aspects of our study relate to the mechanism of CyA induced endothelial injury, the beneficial effects of SRL treatment which maintains ET-1-NO homeostasis and the beneficial effects of BH₄ and BOS treatment, which stabilizes ET-1-NO homeostasis. Our investigations revealed the following observations (summarized in Table 2):

1. CyA results in vasomotor impairment. Individual exposure to BH₄ or BOS attenuates CyA-induced dysfunction while combined therapy with both agents offers superior protection. SRL treatment does not significantly alter the vasomotor properties of the vasculature.

2. SRL, BOS and BH₄ exposure lowers plasma ET-1 levels.

3. CyA exposure significantly increased ETₐ Rc expression while SRL had no effect on ETₐ Rc expression. Concomitant exposure to BH₄ or BOS attenuated the increase in ETₐ Rc expression.

4. BH₄ exposure (with or without concomitant CyA exposure) significantly increased ETₐ Rc expression. BOS attenuated the increase in ETₐ Rc expression associated with BH₄.

5. CyA impaired eNOS protein expression compared to control and SRL. BH₄ partially reversed CyA-induced downregulation. BOS partially abrogated the effects of CyA on eNOS expression. BOS exposure upregulates eNOS expression.

6. CyA exposure led to greater oxidative injury as measured by 8-isoprostane levels compared to both SRL and control. Concomitant therapy with BH₄ or BOS attenuated superoxide production and led to similar 8-isoprostane levels as control. BH₄ and BOS exposure reduced ROS injury and production compared to control.
7. No treatment group had any effect on cytokine production as measured by tissue levels of TNF-α or TGF-β.

6.6.2 CYCLOSPORINE AND VASOMOTOR FUNCTION

Normal vessel function is maintained by the balance between NO and ET-1. Our studies demonstrate that CyA alters both NO and ET-1 regulation. eNOS protein expression was reduced after treatment with CyA. The reduction in eNOS may be a consequence of CyA inhibiting cyclophilin cis-trans peptidyl-prolyl isomerase function resulting in impaired eNOS folding and therefore increased degradation. Lungu et al showed that CyA also inhibits eNOS activity [1074]. Second, although ET-1 levels were not elevated by CyA treatment, ETₐ Rc protein expression in the thoracic aorta was significantly increased with no concomitant change in ETₐ Rc protein expression. Previous studies have demonstrated that CyA-induced injury is caused by ETₐ Rc alterations [738;741]. Our data suggests that CyA also increases ETₐ expression. Hunley et al showed in a rodent model that CyA-induced renal dysfunction was abrogated following treatment with BQ123, an ETₐ Rc antagonist [738]. Takeda et al observed a similar finding in mesangial cells that ET-1 antagonism protects against CyA-induced injury [741]. ETₐ and ETₐ Rc activation on SMCs results in vasoconstriction whereas ETₐ Rc on endothelial cells results in vasodilation at low ET-1 concentrations. Therefore, an increased ETₐ Rc-to-ETₐ Rc ratio results in greater vasoconstriction. Although, CyA does not increase ET-1 levels compared to control, a relative increase may exist since CyA decreased NO levels and therefore a compensatory reduction in ET-1 levels (which was not observed) would be required to maintain normal homeostasis. The normal plasma ET-1 levels seen with CyA treatment may therefore predispose vessels to
vasoconstriction. This was confirmed by our observation that CyA results in greater sensitivity to ET-1 induced vasospasm. Sudhir et al also found no change in ET-1 levels following CyA treatment; however, their study was an acute exposure protocol. In our study we measured ET-1 levels at CyA trough which may also be an ET-1 trough. Plasma CyA concentrations may correlate with changes in plasma ET-1 levels. This hypothesis is supported by the fact that cultured endothelial cells exposed to CyA produce and release ET-1. In renal transplant patients, ET-1 levels are increased three hours after CyA treatment. In a rodent model Takeda et al found increased ET-1 release from rat mesenteric arteries after chronic CyA exposure for 6 weeks. Therefore, ET-1 levels may demonstrate a peak and valley characteristic. Furthermore, prolonged exposure to CyA may gradually increase baseline ET-1 levels. CyA also resulted in a higher SNP ED\textsubscript{50} compared to the other treatment groups indicating impaired cGMP-dependent SMC relaxation. Khalil’s study reinforces our observations by demonstrating that CyA impaired both endothelial dependent and independent vasodilation. Furthermore, CyA-induced impairment in endothelial dependent and independent vasodilation was attenuated by NO augmentation with L-arginine indicating NO dysregulation. In addition, the lack of complete restoration of endothelial function demonstrates the involvement of another mechanism, likely ET-1 dysregulation. Previous studies have suggested that CyA treatment increases free radical production and our study confirmed that oxidative injury occurred following CyA exposure. Using isolated endothelial cell cultures we have showed that CyA exposure greatly increases superoxide production. We therefore speculate that CyA treatment results in functional uncoupling of the eNOS enzyme producing free radicals
instead of NO. Calo et al demonstrated that the NADPH oxidase pathway may also be a source of CyA induced ROS generation [713]. Another potential source of free radicals is iNOS. Our investigations found that iNOS is not induced following CyA treatment. Krauskopf et al also showed that CyA can generate superoxide in smooth muscle which may lead to impaired function [1075]. Hong et al demonstrated in allogenic myoblast transplantation that CyA results in ROS generation via inhibition of the peptidylprolyl-cis-trans-isomerase causing cell injury and death [1077]. Therefore, oxidative injury may account for endothelial and smooth muscle dysfunction observed in our vascular assessments.

6.6.3 RAPAMYCIN AND VASOMOTOR FUNCTION

SRL therapy did not decrease eNOS protein expression as seen following CyA treatment. As a result, there was no impairment in endothelial independent or dependent vasodilation. Naoum et al found that SRL in lipid-fed Apo-E knockout mice increases eNOS protein expression compared to control [776]. They also found that the levels of phosphorylated eNOS were unchanged likely indicating no change in activity [776]. Their study supports our findings in that eNOS protein expression is not downregulated following SRL treatment [776]. Compared to control, SRL resulted in a lower ED$_{50}$ for Ach indicating that SRL may enhance the vasodilatory properties of Ach. This statement is supported by the finding of Corbin et al that SRL led to vasomotor relaxation of rat aortic rings in a dose-dependent fashion [775]. Their study confirms our data showing that at any Ach dose vessel dilation in the SRL group was greater than that of control. Gardiner et al demonstrated in a rodent model that SRL results in reduced renal and mesenteric vascular conductance while CyA increased vasoconstriction [1078]. Furthermore, they showed that CyA
vasoconstriction was attenuated but not completely blocked with pre-treatment with SB 209670 (an ET\textsubscript{A} and ET\textsubscript{B} Rc blocker) [1078]. Their finding supports our results showing that CyA alters ET-1 regulation (increased ET\textsubscript{A} Rc expression as well as a relative increase in ET-1 plasma levels) and that BOS abrogated CyA-induced dysfunction [1078]. The ability of SRL to improve endothelial function may limit and reverse the development of CAV. In fact, Poston et al demonstrated that SRL therapy can reverse the development of CAV [779]. SRL treatment did not result in increased sensitivity to ET-1. In addition, SRL treatment resulted in less oxidative injury compared to CyA. Tunon et al found in rat hepatocytes exposed to LPS that ROS generation was reduced by SRL treatment [1079]. Their study indicates that SRL may result in less oxidative injury not only by the fact that it does not generate ROS but also by inhibiting ROS production [1079]. Therefore, SRL may improve endothelial and smooth muscle function by enhancing NO bioavailability and reducing oxidative injury.

The mechanisms by which SRL preserves endothelial function may also be the mechanism by which it attenuates the development of allograft vasculopathy. Simonson et al have demonstrated that ET-1 inhibition attenuates the development of CAV [104]. Therefore, the ability to lower ET-1 levels may be a mechanism by which SRL attenuates allograft vasculopathy in transplant recipients. SRL treatment preserved eNOS expression compared to CyA and represents another mechanism by which SRL protects against vasculopathy. Lee et al have shown that eNOS protects the aortic allograft from the development of transplant atherosclerosis [1080]. Oxidative stress is well known to result in endothelial damage and atherosclerosis and in these studies, SRL exposure resulted in less free radical injury compared to CyA.
Cytokines have been implicated in the development of CAV. Several investigators demonstrated that TGF-β is associated with the development of CAV [1081-1083]. Our investigations revealed no alterations in TNF-α or TGF-β expression following treatment.

6.6.4 TETRAHYDROBIOPTERIN THERAPY

BH₄ therapy prevented the decrease in eNOS protein expression following CyA treatment. As a result, there was no impairment in endothelial independent or dependent vasodilation. Furthermore, treatment with BH₄ therapy alone did not affect endothelial vasodilation as demonstrated by other investigators [285;1084]. However, BH₄ treatment did result in increased sensitivity to ET-1 despite a decrease in the ETₐ:ETₐRc ratio, likely as a result of greater increased ETₐRc expression on SMCs. Plasma ET-1 levels are reduced following BH₄ treatment which may counteract the effect of ETₐRc upregulation in vivo. In addition, BH₄ treatment resulted in less oxidative injury. The ability of BH₄ to reduce oxygen-derived free radical injury has been demonstrated in other models (41-42). Therefore, BH₄ may improve endothelial and smooth muscle function by enhancing NO bioavailability and reducing oxidative injury. The ability of BH₄ to limit ROS production and oxidative injury indicates the role of eNOS as a source of free radicals.

Our findings that BH₄ improves endothelial function are consistent with studies from several other institutions [1085-1087]. Malo et al demonstrated that treatment with 6-methyltetrahydrobiopterin, a BH₄ analogue, limited endothelial dysfunction seen with left ventricular hypertrophy as a result of aortic banding [1085]. They suggest that endothelial function is preserved via the antioxidant properties of BH₄ [1085]. Fukuda et al demonstrated that hypercholesterolemic patients had impaired coronary endothelial function, which can be restored with BH₄ infusion [1086]. They also found that the effect of
BH₄ could be blocked by a NOS inhibitor indicating that BH₄ improved endothelial function by enhancing NO bioavailability and reducing ROS through eNOS [1086]. A study by Ihlemann et al demonstrated that a glucose challenge results in impaired endothelial dependent vasodilation, which can also be prevented by BH₄ infusion [1087]. Their study was performed in healthy subjects, demonstrating the ability of BH₄ to limit endothelial dysfunction following an acute insult on previously healthy subjects [1087]. This finding has implications for the prevention of endothelial injury in donor cardiac allografts.

6.6.5 BOSENTAN THERAPY

BOS therapy demonstrated significant beneficial vascular protective effects. BOS exposure completely abrogated CyA-induced impairment in endothelial dependent vasorelaxation. Combined ET-1 antagonism and NO augmentation resulted in a higher Emax% compared to control indicating improved endothelial response to Ach. BOS exposure also prevented the increased Ach ED₅₀ seen after CyA treatment and lowered the ED₅₀ to below control levels. These observations indicate an improved vasodilatory response of the vasculature when exposed to BOS.

The effect on endothelial dependent vasodilation may be one mechanism by which ET-1 antagonism reduces the development of allograft vasculopathy. Barton’s data supports our conclusion that ET-1 antagonism preserves NO dependent endothelial function as well as reduces atherosclerosis [601]. Bohm et al also demonstrated improved endothelial function, as assessed by forearm blood flow, after ET-1 blockade with BOS in atherosclerotic patients [1088]. They also observed that BOS had direct vasodilatory properties [1088]. The beneficial properties of BOS are not just limited to the endothelium. We demonstrated that BOS exposure completely abrogates CyA-induced endothelial independent dysfunction.
Combined treatment with BOS and BH$_4$ (with or without CyA) resulted in heightened sensitivity to SNP as indicated by the highest Emax% and lowest ED$_{50}$ compared to all groups. The BOS alone group showed a significant increase in eNOS protein expression which may explain the increased vasodilatory property of the vasculature. Sensitivity to ET-1 induced vasospasm was markedly reduced to below control levels following BOS exposure (all BOS groups). BOS exposure blocked ET-1 Re upregulation seen following CyA (ET$_A$ Re) and BH$_4$ (ET$_B$ Re) treatment. The ability of BOS to prevent ET-1 Re upregulation is the likely mechanism by which it limits CyA and BH$_4$ induced vasospasm. Two-week therapy with BOS reduced ET-1 plasma levels, identifying another potential mechanism by which BOS can decrease the development of CAV. The ability of BOS to reduce plasma ET-1 levels was also observed by Pearl et al in a piglet model of I/R [1049]. CyA-induced ROS production and injury was completely abrogated by BOS exposure indicating a role for ET-1 in the formation of free radicals. Combined therapy further reduced ROS injury compared to control. The ability of BOS to limit oxidative burst and reduce injury is another potential mechanism for endothelial protection.

In summary, we speculate that CyA treatment results in functional uncoupling of the eNOS enzyme producing free radicals instead of NO and that BH$_4$ stabilizes the eNOS complex. As a result of this stabilization, BH$_4$ therapy confers protection against endothelial injury, likely by limiting superoxide production and oxidative injury. BOS preserves endothelial function by upregulating eNOS expression, maintaining normal ET Re levels and reducing ROS production and injury. An effective strategy to treat CyA induced vasomotor dysfunction should include ET-1 antagonism in addition to functional coupling of eNOS. This may be achieved by using BOS for ET-1 antagonism and BH$_4$ for stabilizing
the eNOS complex and reducing free radical production. Combined therapy may provide the optimal strategy as BOS limits the deleterious effects of BH$_4$ and BH$_4$ augments the protective effects of BOS. Our findings provide potential mechanisms for the development of CyA induced hypertension as well as a direct mechanism by which CyA may lead to CAV.

SRL may prove to be an alternative therapy to CyA for preserving vasomotor function. Poston et al using a rodent heterotopic heart transplant model demonstrated the ability of SRL to reverse chronic graft vascular disease [779]. Their study describes the beneficial effects of SRL on vasculopathy but did not evaluate the underlying mechanism of benefit. Their study did reveal that CAV occurs in the absence of myocardial rejection, suggesting that SRL’s protective effects are likely not mediated by its immunosuppressive activity. Our studies suggest that alterations in NO-ET-1 homeostasis plays a critical role in the development of CAV. Using the same model, Yamaguchi et al demonstrated that ET-1 blockade reduces CAV, supporting our hypothesis that ET-1 is involved in the pathogenesis of CAV [103]. In this study, ET-1 production was inhibited pre-operatively prior to transplantation with the use of anti-sense oligodeoxynucleotides resulting in a 7 to 10 day suppression of ET-1. Our present study demonstrated that ET-1 levels are relatively elevated by CyA and that ET$_A$ receptor upregulation lasts for at least two weeks indicating that chronic therapy may be required for enhanced protection against CyA induced CAV. Verrier et al. demonstrated in a rodent model of orthotopic aortic allograft transplantation that I/R results in endothelial injury leading to the development of CAV [8]. Therefore, Yamaguchi’s study may in fact demonstrate the protective effect of acute ET-1 blockade in limiting I/R induced CAV rather than drug induced CAV. Both of these studies support our
hypothesis that by maintaining vascular homeostasis SRL may prevent CAV and that ET-1 antagonism may provide additional benefit.

In conclusion, we have made the following observations; 1. CyA results in alteration of both NO and ET-1 regulation leading to impairment of vasodilation, increased sensitivity to vasospasm and increased oxidative injury. 2. BH$_4$ can stabilize the eNOS complex and attenuate the deleterious effects on CyA. 3. BH$_4$ therapy may be associated with increased sensitivity to ET-1. 4. SRL preserves the eNOS complex, lacks the deleterious effects of CyA on the endothelium, and decreases ET-1 levels. 5. BOS treatment prevents CyA-induced vasomotor dysfunction. 6. ET-1 antagonism in addition to NO augmentation offers superior protection without deleterious side-effects.

6.7 SUMMARY OF INVESTIGATIONS AND ORIGINAL CONTRIBUTIONS

These investigations evaluate the role of ET-1 in endothelial dysfunction, H/R and immunosuppressive drug injury. We also investigated the potential beneficial effect of the ET-1 antagonist, bosentan, at protecting the endothelium from ET-1, H/R injury and immunosuppressive drug injury. In addition, we examined whether BOS possessed PC like properties. ET-1 was found to be deleterious to the endothelium and vasculature under both normoxic and hypoxic conditions. Exposure to BOS limited ET-1, H/R and immunosuppressive drug injury. We also demonstrated the role of PKC and PKB in endothelial dysfunction and cell death. The results of our studies yielded several original contributions to the literature:

1. Elevated ET-1 levels impair NO homeostasis in *human* endothelial cells.
   a) ET-1 inhibits PKC$\lambda$ decreasing NO production
   b) ET-1 activates PKC$\delta$ decreasing NO production.
c) ET-1 results in eNOS downregulation by a PKC dependent pathway.

d) ET-1 increases the eNOS M/C ratio via PKC modulation and PKB inhibition.

2. ET-1 induces the production of ROS.

   a) ET-1 increases eNOS and NAPDH oxidase ROS generation.

   b) ET-1 results in eNOS uncoupling.

   c) ET-1 induced PKCδ and PKCε activation increases ROS production.

   d) ET-1 inhibition of Akt/PKB increases ROS formation.

3. H/R impairs endothelial function.

   a) ET-1 is a key player in H/R induced endothelial dysfunction.

   b) ET-1 exposure during H/R exacerbates H/R induced dysfunction.

   c) H/R decreases eNOS expression through PKC modulation.

   d) H/R increases the eNOS M/C ratio through modulation of PKC and PKB activity.

4. H/R increases ROS generation.

   a) ET-1 is involved in H/R ROS generation and exacerbates H/R induced ROS formation.

   b) H/R releases ROS by PKCδ and PKCε activation and PKB inhibition.

   c) H/R induced eNOS and NADPH oxidase derived ROS leads to apoptosis.

5. H/R increases cell death.

   a) H/R mediates cell death via apoptosis and not via necrosis or autophagy.

   b) H/R activates both the intrinsic and extrinsic apoptotic pathways.

   c) ET-1 plays a role in H/R induced cell death.
d) ET-1 exposure accentuates H/R apoptosis.

e) H/R regulates cell death by increasing PKCδ and PKCε activity and inhibiting PKCλ and Akt/PKB activation.

6. CyA impairs vasomotor function.
   a) CyA impairs endothelial dependent and independent vasorelaxation
   b) CyA increases ET-1 vasospasm.
   c) CyA results in a relative increase in ET-1 levels.
   d) CyA downregulates eNOS while upregulating ET₄ receptor expression.
   e) CyA increases ROS production and oxidative injury.

7. SRL maintains vasomotor function.
   a) SRL does not impair normal vasculature vasodilation or vasoconstriction, compared to CyA.
   b) SRL maintains eNOS and ET₄ receptor expression compared to CyA.
   c) SRL reduces ET-1 plasma levels

8. BH₄ therapy limits CyA induced dysfunction.
   a) Prevented CyA induced endothelial dysfunction.
   b) Increased vessel sensitivity to vasospasm.
   c) Reduced ET-1 plasma levels.
   d) Decreased ROS production and oxidative injury.
   e) BH₄ reduced endothelial cell ROS generation, injury and death.

9. BOS therapy improved endothelial function and survival.
   a) BOS improved endothelial cell NO production.
   b) BOS can modulate PKC and PKB activity.
c) BOS reduced endothelial cell ROS generation.
d) BOS possesses PC like properties.
e) BOS abrogates CyA induced vasomotor dysfunction.
f) BOS prevents CyA generated ROS and oxidative injury.
g) BOS decreases plasma ET-1 levels.
h) BOS abrogated the negative effects of BH₄.

In summary, ET-1 plays an important role in endothelial homeostasis under normoxic and hypoxic condition by altering NO and ROS production as well as cell death. ET-1 is also involved in immunosuppressive drug injury. BOS proved to possess endothelial protective effects under various conditions and may be a powerful treatment strategy to reduce allograft failure and CAV. Finally, our investigations revealed that protein kinase modulation (specifically PKC and PKB) can protect the endothelium from various insults.

**6.8 STUDY LIMITATIONS**

Our study provides evidence that ET-1 impairs NO homeostasis in an *in vitro* model through a PKC dependent pathway. Our use of a supraphysiologic dose of ET-1 may not be entirely representative of what occurs *in vivo*. Although our study demonstrated the role of PKC and Akt/PKB in ET-1 induced endothelial injury, it does not exclude the involvement of alternative signaling pathways. In addition, there are differences between venous and arterial cells in vivo but in vitro, most endothelial cells de-differentiate into a common phenotype after passaging.

This thesis was also designed to investigate the direct effect of CyA and SRL on vascular function in the absence of an immune response or a period of ischemia and
reperfusion. Clearly, the effects of the latter two variables will need further assessment in a heterotopic transplant model similar to that of Poston and Yamaguchi. In addition, we evaluated changes in aortic tissue as opposed to coronary arteries. Although the responses are likely consistent, it is possible that both CyA and SRL exert differential effects on coronary vasculature than seen in thoracic aorta. However, the macrovascular effects of these agents have important clinical implications for the development of postoperative renal insufficiency and hypertension. Lastly, we did not evaluate the effects of combination therapy with both CyA and SRL, a common clinical strategy.

While the present thesis does confirm a beneficial effect of combined therapy with BH4 and Bosentan over 2 weeks, it is unclear if prolonged therapy is necessary to achieve protection against the development of CyA induced hypertension, nephropathy or vasculopathy. A direct comparison of acute vs chronic therapy is required to define the optimal timing of treatment. Nevertheless, both BH4 and Bosentan have been employed clinically with a favourable side effect profile. Thus, the potential for prolonged protection would favour long term therapy with both agents in all solid organ transplant recipients receiving cyclosporine immunosuppression.

6.9 FUTURE AREAS OF RESEARCH

The results of these investigations provide a potential mechanism of ET-1 induced endothelial dysfunction, H/R cell death and immunosuppressive drug injury. We provided a rationale for the use of BOS or protein kinase modulation in preventing endothelial injury under various insults. The clinical use of BOS in heart failure patients was associated with an increased risk of sudden death likely due to hypotension. Our study reveals that PKC and/or PKB modulation may be an attractive alternative to restore
endothelial function. Heart failure patients treated with new isoform specific PKC modulators may benefit from BOS like protection without the risk of hypotension induced arrhythmias.

We provide evidence that BOS and PKC as well as PKB modulation can improve endothelial cell function and survival after H/R. Several clinical studies are currently evaluating the effects of ET-1 antagonism and isoform specific PKC modulation at preventing allograft failure. These studies focus on myocardial protection. Our data suggest that endothelial protection can also be achieved. Therefore, future clinical studies are required to substantiate our in vitro findings. We are confident that our in vitro observations have the potential for clinical applicability as we have previously demonstrated in a porcine model of transplantation that BOS can improve allograft function (myocardial and endothelial) [17]. I/R injury at the time of transplantation is also associated with the development of CAV [8]. Therefore, limiting endothelial damage at the time of transplantation may reduce the burden of CAV. Clinical studies will be required to correlate the benefit of BOS and protein kinase modulation at the time of organ retrieval and transplantation with the decreased development of CAV.

Immunosuppressive drugs have revolutionized clinical organ transplantation. However, these agents have been demonstrated to result in endothelial damage and CAV. We provide evidence for the mechanisms behind the pathogenesis of CAV by CyA. We demonstrated that ET-1 antagonism as well as NO supplementation limits vascular injury. Sekhon et al also showed that combined therapy with ET-1 reduction and NO augmentation offers superior protection to single agent therapy [1047]. A transplant model is required to confirm the benefits of our treatment strategy on reducing CAV.

...
addition targeted pharmacologic interventions with protein kinase modulators may provide more specific and superior protection. Our studies also provide evidence for the use of antioxidant therapy in limiting H/R and immunosuppressive drug injury. In addition, we found that SRL treatment maintains normal vascular homeostasis and may represent an attractive alternative to CyA therapy.

We demonstrated that BOS possesses PC like properties. Whether this effect occurs in vivo remains to be determined. The importance of limiting endothelial injury is paramount in the cardiovascular world since endothelial dysfunction is associated with atherosclerosis, CAV and allograft failure. Strategies to limit such injury can reduce the burden of disease and limit mortality for the number one killer in the Western World. In addition, improving endothelial protection during cardiac transplantation may increase the donor pool as the use of marginal donors can be expanded. Improved perioperative and postoperative endothelial protection will have a great impact on long term clinical outcomes following heart transplantation.
CHAPTER SEVEN

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REFERENCES


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CHAPTER EIGHT

FIGURES AND TABLES
Figure 2. Maximal intimal thickness one year after cardiac transplantation. Pravastatin significantly attenuated intimal proliferation during the first year following transplantation.
Figure 3: Mechanism of preconditioning. This figure demonstrates the central role that PKC can play in preconditioning. PKC can be activated by preconditioning triggers and be responsible for signal transduction that eventually results in an end-effector effect. Adapted from Gross et al [2].
Figure 4: Two step reaction of nitric oxide formation. Step 1, L-arginine is converted to $N^G$-hydroxyl-L-arginine. Step 2, $N^G$-hydroxyl-L-arginine is converted to L-citrulline and nitric oxide.
Figure 5: Classical PKC Activation. Classical PKC activation occurs by binding of a ligand to its G-protein coupled receptor leading to PLC activation and the formation of IP3 and DAG. IP3 increases cytosolic calcium concentrations. Both calcium and DAG lead to PKC activation.
Figure 6: Schematic representation of PKC structure. PKC is composed of a regulatory domain and a catalytic domain. The regulatory domain determines DAG and or PS binding. The regulatory domain also contains the autoinhibitory pseudosubstrate. The catalytic domain contains the active site. When PKC is activated the active site performs the kinase function.
cAMP dependent protein kinase

Regulatory Subunit

Autophosphorylation site/autoinhibitory region

<table>
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<th>143</th>
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<tr>
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Dimerization domain

Catalytic Subunit

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<td>Mg²⁺/ATP binding site</td>
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<tr>
<td>Protein substrate interaction</td>
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Figure 7: Schematic representation of PKA structure. PKA is composed of two subunits: the regulatory subunit and the catalytic subunit. The regulatory subunit allows for dimerization and inhibition of the catalytic subunit. The catalytic subunit confers the kinase function and does not require activation for activity. The catalytic subunit is constitutively active in the monomeric form and therefore dimerization is the only method of inhibition.
PKA Activation

Figure 8: Schematic representation of PKA activity. PKA is in a tetrameric inactive state. Upon activation of cAMP, two molecules of cAMP bind to the regulatory subunit and decrease affinity of the regulatory subunit for cAMP. Two more cAMP molecules are required to disassociate the catalytic (CS) subunits which do not require activation for kinase activity.
ET-1 Structure

Figure 9: Schematic representation of ET-1 structure. ET-1 is a 21 amino acid protein with two disulfide bonds between cysteine 3 and 11 as well as cysteine 1 and 15. These bonds are important for ET-1 activity. The terminal amino acid is tryptophan which is also crucial for activity.
Figure 10: A, Western Blot for plasma calcium-ATPase demonstrated no protein presence in the cytosolic fraction indicating no membrane contamination of the cytosolic fraction. B, Western blot analysis for β-actin showing no protein presence in the membrane fraction revealing no cytosolic contamination of the membrane fraction.

Legend: M: membrane fraction; C: cytosolic fraction.
Figure 11: Dose-response effect of ET-1 treatment on HSVEC NO production. ET-1 resulted in a concentration-dependent decrease in NO production in HSVEC at concentrations above 1nM. Treatment with 1nM concentration of ET-1 significantly increased NO production. However, exposure to ET-1 concentrations below 1nM had no effect on NO production.

Legend: Con: Control; ET: Endothelin-1; NO: Nitric oxide; HSVEC: Human saphenous vein endothelial cell.
Figure 12: NO production in HSVEC following treatment for 30 minutes and 24 hours. The presence of ET-1 significantly reduced NO production following 30 minutes with a greater inhibition after 24 hours. Exposure to the PKC inhibitors calphostin C and chelerythrine significantly lowered NO release from HSVECs. However, co-incubation of ET-1 with PKC inhibitors demonstrated no added reduction in NO levels. HSVEC exposed to PMA demonstrated an increased NO production compared to control and significantly abrogated ET-1 induced effects. BOS treatment was effective at blocking ET-1 effects at both 30 minutes and 24 hours.

Legend: NO: Nitric Oxide; HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan.
Figure 13: NO production in HSVEC following treatment for 24 hours. The presence of ET-1 significantly reduced NO production following 24 hours. Exposure to DI did not effect NO production while ZI and EI reduced NO release. Co-incubation of ET-1 with ZI or EI further decreased NO levels. DI exposure limited ET-1 effects on NO impairment.

Legend: NO: Nitric oxide; HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; ZI: PKCλ inhibitor, DI: Rottlerin; EI: PKCε V1-2.

![Graph showing NO Production](image)
Figure 14: NO production in HSVEC following treatment for 30 minutes and 24 hours. The presence of ET-1 significantly reduced NO production following 30 minutes with a greater inhibition after 24 hours. Exposure to the PKA inhibitor H89 did not alter NO production. HSVEC exposed to the PKA agonist FOS demonstrated no change in NO production compared to control. Modulation of PKA activity did not alter ET-1 induced impairment of NO homeostasis.

Legend: NO: Nitric Oxide; HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; FOS: Forskolin (PKA agonist).
Figure 15: NO production in HSVEC following treatment for 30 minutes and 24 hours. The presence of ET-1 significantly reduced NO production following 30 minutes with a greater inhibition after 24 hours. Exposure to the Akt/PKB inhibitor SH5 reduced NO production. Co-incubation of SH5 with ET-1 demonstrated a significant additive reduction in total nitrite levels.

Legend: NO: Nitric Oxide; HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1.
Figure 16: ROS production in HSVEC following treatment for 24 hours. The presence of ET-1 significantly increases ROS production. Exposure to BOS attenuates ET-1 induced effects. PKC inhibition decreases ROS production while PMA increased ROS release. SH5 treatment results in free radical generation. PMA and SH5 aggravated ET-1 induced ROS release. PKA modulation had no effect on ROS production.

Legend: ROS: Reactive oxygen species; HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; BOS: Bosentan; Cal: Calphostin C; Chel: Cheletherine; FOS: Forskolin (PKA agonist).
Figure 17: ROS production in HSVEC following treatment for 24 hours. ZI and PKCε V1-2 exposure inhibited ROS production while DI did not demonstrate any difference compared to control. Co-incubation of ET-1 with PKCε V1-2 or DI resulted in a significant reduction in intracellular ROS production compared to ET-1 alone. Co-incubation with ZI did not led to any significant changes compared to ET-1.

Legend: ROS: Reactive oxygen species; HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; ZI: PKCα inhibitor; DI: Rottlerin; EI: PKCε V1-2.
Figure 18: ROS production in HSVEC following treatment for 24 hours. In control cells sepiapterin (NOS cofactor), L-NAME (NOS inhibitor), apocynin (NADPH oxidase inhibitor) and tiron (SOD anion) did not significantly lower ROS production. Tempol (SOD mimetic) decreased ROS production. The use of sepiapterin, L-NAME, apocynin and tempol reduced ET-1 derived ROS, while tiron had no significant effect on ET-1 induced ROS production.

Legend: ROS: Reactive oxygen species; HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; H2O2: hydrogen peroxide; Sep: Sepiapterin; TI: Tiron; TE: Tempol.
Figure 19: A, Western Blot for PKCα. PKCα was expressed solely on the membrane fraction. No significant differences were seen between groups. B, Western Blot for PKCι. PKCι was expressed solely on the cytosolic fraction. No significant differences were seen between groups.
Legend: Control; ET: Endothelin-1; BOS: Bosentan; +’ve: Positive control; M: membrane fraction; C: cytosolic fraction.
Figure 20: PKC\(\delta\) Translocation. A. Western Blots for PKC\(\delta\) protein expression demonstrating significant differences between groups. B, Thirty minute exposure to ET-1 significantly translocated PKC\(\delta\) to the membrane compared to control. Treatment with PKC inhibitors significantly reduced the M/C ratio suggesting an increased cytosolic expression of PKC\(\delta\), whereas PMA resulted in a significantly higher M/C ratio in the control group and not in the ET-1 exposed cells. Bosentan treatment abrogated ET-1 induced translocation.

Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); +ve: Positive control.
Figure 21: PKC\(\delta\) Translocation. A. Western Blots for PKC\(\delta\) protein expression demonstrating significant differences between groups. B. 24 hour exposure to ET-1 caused PKC\(\delta\) translocation. PKC inhibition for 24 hour reduced while PMA increased the M/C ratio only in the control group. Bosentan exposure attenuated ET-1 induced effects.

Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); +’ve: Positive control.
PKCε Translocation (30 min)

Figure 22: PKCε Translocation. A. Western Blots for PKCε protein expression demonstrating significant differences between groups. B. PKCε was significantly translocated to the membrane following ET-1 treatment compared to control. Treatment with PKC inhibitors significantly reduced the M/C ratio in ET-1 exposed cells while having no effect on the control group. PMA resulted in a significantly higher M/C ratio in the control group and not in the ET-1 exposed cells. Bosentan exposure attenuated ET-1 induced translocation. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); +ve: Positive control.
Figure 23: PKε Translocation. A. Western Blots for PKε protein expression demonstrating significant differences between groups. B, 24 hour exposure to ET-1 caused PKε translocation. PKC inhibition for 24 hour lowered the M/C ratio only in the ET-1 group while PMA increased the M/C ratio only in the control group. HSVEC exposed to Bosentan blocked ET-1 induced effects.

Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); +’ve: Positive control.

Group p<0.0001
* p<0.05 vs. Con
# p<0.05 vs. ET

PKε Translocation (24 hour)

<table>
<thead>
<tr>
<th>M/C Ratio</th>
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<th>Chel</th>
<th>PMA</th>
<th>BOS</th>
<th>SH5</th>
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<td>ET</td>
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Graph showing bar chart with M/C Ratio on the y-axis and various treatments on the x-axis.
Figure 24: PKCλ Translocation. A. Western Blots for PKCλ protein expression demonstrating significant differences between groups. B, The presence of ET-1 for 30 minutes resulted in a significant lowering of the PKCλ M/C ratio. Treatment with PKC inhibitors significantly reduced the M/C ratio in the control group while having no effect on the ET-1 treated cells. PMA resulted in a significantly higher M/C ratio in both the control and ET-1 groups. BOS exposure in addition to attenuating ET-1 induced effects also resulted in a significantly higher translocation of PKCλ.

Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); +ve: Positive control.
Figure 25: PKCλ Translocation. A. Western Blots for PKCλ protein expression demonstrating significant differences between groups. B, Exposure of HSVEC to ET-1 for 24 hours caused a significant lowering of the PKCλ M/C ratio. PKC inhibition for 24 hours lowered the M/C ratio while PMA increased the M/C ratio. HSVEC exposed to BOS blocked ET-1 induced effects while treatment of cells with BOS alone also resulted in an increased PKCλ M/C ratio. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); +ve: Positive control.

Group p<0.0001
* p<0.05 vs. Con
# p<0.05 vs. ET
Figure 26: HSVEC PKC activity. ET-1 caused a significant decrease in PKC activity following 30 minute exposure with a further reduction after 24 hours. Exposure to PKC inhibitors (Cal and Chel) resulted in a lowering of HSVEC PKC activity while PMA increased activity. BOS exposure blocked ET-1 reduction in PKC activity and increased PKC activity compared to control.

Legend: HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chelerythrine; BOS: Bosentan.
Figure 27: HSVEC PKC activity. Akt/PKB inhibition with SH5 slightly reduced PKC activity following 30 minutes with no further reduction after 24 hours. Co-incubation with ET-1 resulted in a significant reduction compared to control but not compared to ET-1 at 30 minutes and 24 hours. PKA modulation with either FOS or H89 did not result in any significant changes in PKC activity and did not alter the ET-1 induced reduction in PKC activity.

Legend: HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; FOS: Forskolin (PKA agonist).
Figure 28: HSVEC PKC activity. ZI specifically inhibited PKCλ in our endothelial cells. ET-1 exposed cells demonstrated a significant reduction in PKCλ activity compared to control but was significantly higher than PKCλ activity with ZI ($F=53.81$, $p<0.0001$). PMA resulted in significant activation of PKCλ.

Legend: HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; ZI: PKCl inhibitor; Cal: Calphostin C; Chel: Chelerythrine.
Figure 29: HSVEC PKA activity. ET-1 exposure regardless of duration did not alter PKA activation compared to control. Modulation of PKC activity also failed to change cellular PKA activation. HSVEC treated with BOS did not modify PKA activity.

Legend: HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan.
Figure 30: HSVEC PKA activity. Modulation of PKB activity failed to change cellular PKA activation. The PKA agonist FOS led to PKA activation while therapy with the PKA antagonist H89 resulted in decreased activity compared to all other groups. There was a time-dependent decrease in activity following H89 treatment and a time-dependent increase following FOS exposure.

Legend: HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; FOS: Forskolin (PKA agonist).
Figure 31: Akt Phosphorylation. A. i-iii) Western blot for total and phospho-Akt expression demonstrated significant differences between groups at 30 minutes. B i) Quantitative Western blot analysis of phospho-Akt/Total Akt ratio. ET-1 reduces both serine and threonine phosphorylation compared to control.

Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; +‘ve: Positive control.
Figure 32: Akt Phosphorylation. A. i-iii) Western blot for total and phospho-Akt expression demonstrated significant differences between groups at 24 hours. B i) Quantitative Western Blot analysis of phospho-Akt/Total Akt ratio. ET-1 reduces both serine and threonine phosphorylation compared to control.

Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; +’ve: Positive control.
Figure 33: Caveolin-1 protein expression. A. Western blot for caveolin-1 expression demonstrated no differences between groups following 30 minutes. B. Western blot for caveolin-1 expression demonstrated no differences between groups after 24 hour exposure to treatment. C. Western blot for β-actin expression demonstrated no significant differences between groups.

Legend: +ve: iNOS positive control. Con: Control; ET: endothelin-1; BOS: Bosentan; Cal: Calphostin C; Chel: Chelerythrine; FOS: Forskolin (PKA agonist).
Figure 34: iNOS protein expression. A. Western blot for iNOS expression demonstrated no induction following 30 minutes. B. Western blot for iNOS expression demonstrated no induction following 24 hours of exposure to treatment.
Legend: MW: Molecular Weight; +’ve: iNOS positive control.
Figure 35: eNOS protein expression. A. i-ii) Western blot for eNOS expression demonstrated no significant differences between groups at 30 minutes. iii) Western blot for β-actin expression demonstrated no significant differences. B i) Quantitative Western blot analysis of eNOS protein expression after 30 minutes of treatment demonstrated no significant differences between groups.

Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); +’ve: Positive control.
Figure 36: eNOS protein expression A. i-iii) Western blot for eNOS following 24-hour exposure demonstrated significant differences between groups iv) Western blot for β-actin expression demonstrated no significant differences. B i) Quantitative Western blot analysis of eNOS protein expression after 24 hours of treatment demonstrated that ET-1 exposure downregulates eNOS protein expression compared to control and BOS abrogates the effects of ET-1. PKC inhibition with Cal or Chel decreased eNOS expression while the PKC agonist PMA increased expression. PKA and PKB modulation had no effect on eNOS expression.

Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); +’ve: Positive control.
Figure 37: eNOS Localization. A. Western Blot depicting membrane and cytosolic eNOS protein expression. B. Exposure of HSVEC to ET-1 for 30 minutes caused a significant increase in the eNOS M/C ratio. PKC inhibition for 30 minutes with Cal or Chel increased the M/C ratio while the PKC agonist PMA decreased the M/C ratio. BOS exposure blocked ET-1 induced effects. SH5 increased ET-1 induced eNOS membrane sequestration.

Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); +’ve: Positive control.

- Group $p<0.0001$
- * $p<0.05$ vs. Con
- # $p<0.05$ vs. ET
Figure 38: eNOS Localization. A. Western Blot depicting membrane and cytosolic eNOS protein expression. B. Exposure of HSVEC to ET-1 for 24 hours caused a significant increase in the eNOS M/C ratio. PKC inhibition for 24 hours increased the M/C ratio while PMA decreased the M/C ratio. BOS exposure blocked ET-1 effects. SH5 increased ET-1 induced eNOS membrane sequestration.

Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); +’ve: Positive control.
Figure 39: Cell Viability. H/R decreased cell viability. ET-1 exposure further reduced cell viability. BOS therapy improved HSVEC tolerance to both H/R and ET-1 induced injury. Exposure to zvad, a caspase inhibitor, during hypoxia alone attenuated the effects of ET-1 and only reduced the effects of H/R when given during both hypoxia and reperfusion.

Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; BOS: Bosentan; Hyp: Hypoxia; H/R: Hypoxia and reperfusion.
Figure 40: Cell Viability and PKC Modulation. PKC inhibition during hypoxia with Cal or Chel demonstrated a significant reduction in viability compared to control after 4 hours of reperfusion with a further decrease after 24 hours. PMA led to a decreased cell viability compared to control at both one and 24 hours of reperfusion. Co-incubation of Cal, Chel or PMA with ET-1 led to a further impairment in cell viability compared to control, Cal, Chel or PMA.

Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine.
Figure 41: Cell Viability and PKA/PKB Modulation. Inhibition of Akt/PKB with SH5 significantly reduced viability compared to control (4 and 24 hours) and ET-1 (24 hours). ET-1 co-incubated with SH5 for 4 hours impaired viability compared to SH5 alone, but by 24 hours there were no differences between these two groups. Altering PKA activity with FOS or H89 had no significant impact on H/R nor ET-1 induced injury.

Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; FOS: Forskolin (PKA agonist).
Figure 42: Cell Viability and Treatment During Reperfusion. BOS given after hypoxia restored viability to near normoxic levels. BOS exposure during H/R maintained cell viability. Extreme reduction in viability was seen following ET-1 exposure during H/R. BOS was able to partially attenuate SH5 induced impairment and completely block the synergistic effect of ET-1 on SH5-induced injury.

Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; BE: BOS+ET; Con/BOS: treatment with Bosentan (BOS) during reperfusion; ET/BOS: Hypoxic treatment with ET and treatment with BOS during reperfusion; ET/ET: ET treatment during hypoxia and reperfusion (H/R); BOS/BOS: BOS during both hypoxia and reperfusion; BE/BE: BE during both hypoxia and reperfusion.
Figure 43: Homogeneous Caspase Activity. Following hypoxia significant activation of homogeneous caspases (3, 6, 7) was observed. Reperfusion resulted in a time dependent increase in caspase activity. ET-1 exposure during hypoxia enhanced caspase activation following H/R. BOS therapy abrogated both H/R and ET-1 induced effects. The caspase inhibitor zvad attenuated caspase activation following H/R.

Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; BOS: Bosentan; Hyp: Hypoxia; H/R: Hypoxia and reperfusion.
Figure 44: Homogeneous Caspase Activity. Hydrogen peroxide treatment during hypoxia increased caspase activation. When hydrogen peroxide was added during hypoxia and reperfusion further caspase activation occurred.

Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; H2O2: Hydrogen peroxide; H/R: Hypoxia and reperfusion.
Figure 45: Homogeneous Caspase Activity. PKC inhibition resulted in greater caspase activation compared to control following each time point. PKC activation also resulted in greater caspase activation compared to control. Co-incubation with ET-1 resulted in an added increase in caspase activity.

Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; Hyp: Hypoxia; H/R: Hypoxia and reperfusion.
Figure 46: Homogeneous Caspase Activity. PKA modulation did not alter the HSVEC response to H/R or ET-1 induced caspase activation. Akt/PKB inhibition significantly increased caspase activation following H/R. Co-incubation of SH5 with ET-1 further increased caspase activation. Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; FOS: Forskolin (PKA antagonist); Hypoxia; H/R: Hypoxia and reperfusion.
Figure 47: Homogeneous Caspase Activity. PKCλ inhibition during hypoxia significantly increased caspase activation compared to H/R alone while DI and EI significantly reduced caspase activity during all time points. ZI incubation during hypoxia and reperfusion showed increased caspase activity compared to control after one hour of reperfusion with no differences at 24 hours. DI and EI therapy during hypoxia significantly reduced ET-1 induced caspase activation following both hypoxia and reperfusion. Further protection against the development of apoptosis was seen when DI and EI were given during hypoxia and reperfusion. ET-1 co-incubation with ZI during hypoxia or H/R resulted in an additive increase in caspase activity.

Legend: No inh: No inhibitor; ZI: PKCλ Inhibitor; DI: Rottlerin; EI: PKCε inhibitor; Con: Control; ET: Endothelin-1; H/R: Hypoxia and reperfusion.
Figure 48: Homogeneous Caspase Activity. Antioxidant (AO) therapy during hypoxia reduced caspase activation compared to No AO. Reperfusion with AO further decreased caspase activation. Greater reduction in caspase activity was observed with Sep (NOS cofactor), L-NAME (NOS inhibitor) and apocynin (NADPH oxidase inhibitor) compared to TE (SOD mimetic) and TI (SOD anion). Sep and L-NAME offered better protection versus apocynin. Legend: No AO: No antioxidants; Con: Control; Sep: sepiapterin; TE: Tempol; TI: Tiron; H/R: Hypoxia and reperfusion.
Figure 49: Homogeneous Caspase Activity. Antioxidant (AO) therapy during hypoxia in the ET-1 group reduced caspase activation compared to No AO. Reperfusion with AO further decreased caspase activation. Greater reduction in caspase activity was observed with Sep (NOS cofactor), L-NAME (NOS inhibitor) and apocynin (NADPH oxidase inhibitor) compared to TE (SOD mimetic) and TI (SOD anion). Sep and L-NAME offered better protection versus apocynin. Legend: No AO: No antioxidants; ET: Endothelin-1; Sep: sepiaapterin; TE: Tempol; TI: Tiron; H/R: Hypoxia and reperfusion.
Figure 50: Homogeneous Caspase Activity. Antioxidant (AO) therapy with BOS offered an approximate 10% decrease in apoptosis during reperfusion compared to BOS alone.

Legend: No AO: No antioxidants; BOS: Bosentan; Sep: sepiapterin (NOS cofactor); TE: Tempol (SOD mimetic); TI: Tiron (SOD anion); H/R: Hypoxia and reperfusion.
Figure 51: Homogeneous Caspase Activity. Even in the presence of ET-1, antioxidant (AO) therapy with BOS resulted an approximate 10% decrease in apoptosis during reperfusion compared to BOS alone.

Legend: No AO: No antioxidants; BOS+ET: Bosentan+endothelin-1; Sep: sepiapterin (NOS cofactor); TE: Tempol (SOD mimetic); TI: Tiron (SOD anion); H/R: Hypoxia and reperfusion.
Homogeneous Caspase Activity

Treatment during hypoxia and reperfusion (H/R)

Figure 52: Homogeneous Caspase Activity. BOS therapy during hypoxia and reperfusion attenuated the effects of H/R, ET and SH5 on caspase activation. ET-1 treatment during both H and R enhanced caspase activity after H/R compared to all other treatment groups.

Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; BOS: Bosentan; BE: BOS + ET; Con/BOS: treatment with BOS during reperfusion; ET/BOS: Hypoxia with ET and treatment with BOS during reperfusion; ET/ET: ET during hypoxia and reperfusion (H/R); BOS/BOS: BOS during H/R; BE/BE: BE during H/R.
Figure 53: Caspase 8 Activity. Following hypoxia, a significant activation of caspase 8 was observed. Reperfusion resulted in a time dependent increase in caspase 8 activity. ET-1 exposure during hypoxia enhanced caspase 8 activation following H/R. BOS therapy abrogated both H/R and ET-1 induced activation. The caspase inhibitor zvad attenuated caspase 8 activation following H/R.

Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; BOS: Bosentan; Hyp: Hypoxia; H/R: Hypoxia and reperfusion.
Figure 54: Caspase 8 Activity. PKC inhibition and activation resulted in greater caspase 8 activation following H/R than control. Co-incubation with ET-1 resulted in an added increase in caspase 8 activity. A reperfusion time dependent increase in caspase 8 activity was observed.

Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; Hyp: Hypoxia; H/R: Hypoxia and reperfusion.
Figure 55: Caspase 8 Activity. PKA modulation did not alter the response of HSVEC to H/R or ET-1 induced caspase 8 activation. Akt/PKB inhibition significantly increased caspase 8 activation following H/R. Co-incubation of SH5 with ET-1 further increased caspase 8 activation. Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; FOS: Forskolin (PKA agonist); Hypoxia; H/R: Hypoxia and reperfusion.
Figure 56: Caspase 8 Activity. BOS therapy during hypoxia and reperfusion attenuated the effects of H/R, ET and SH5 on caspase 8 activation. ET-1 treatment during both H and R enhanced caspase 8 activity after H/R compared to all other treatment groups.

Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; BOS: Bosentan; BE: BOS+ET; Con/BOS: treatment with BOS during reperfusion; ET/BOS: Hypoxia with ET and treatment with BOS during reperfusion; ET/ET: ET during hypoxia and reperfusion (H/R); BOS/BOS: BOS during H/R; BE/BE: BE during H/R.
Figure 57: Caspase 9 Activity. Following hypoxia, a significant activation of caspase 9 was observed. Reperfusion resulted in a time dependent increase in caspase 9 activity. ET-1 exposure during hypoxia enhanced caspase 9 activation following H/R. BOS therapy abrogated both H/R and ET-1 induced effects. The caspase inhibitor zvad given during hypoxia attenuated caspase 9 activation following H/R with a further reduction in caspase 9 activity when zvad was administered throughout hypoxia and reperfusion.

Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; BOS: Bosentan; Hyp: Hypoxia; H/R: Hypoxia and reperfusion.
Figure 58: Caspase 9 Activity. PKC inhibition and activation resulted in greater caspase 9 activation following H/R than control. Co-incubation with ET-1 resulted in an added increase in caspase 9 activity. A reperfusion time dependent increase in caspase 9 activity was also observed. Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; Hyp: Hypoxia; H/R: Hypoxia and reperfusion.
Figure 59: Caspase 9 Activity. PKA modulation did not alter H/R or ET-1 induced caspase 9 activation. Akt/PKB inhibition significantly increased caspase 9 activation following H/R. Coincubation of SH5 with ET-1 further increased caspase 9 activation.

Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; FOS: Forskolin (PKA agonist); Hypoxia; H/R: Hypoxia and reperfusion.
Figure 60: Caspase 9 Activity. BOS therapy during hypoxia and reperfusion attenuated the effects of H/R, ET and SH5 on caspase 9 activation. ET-1 treatment during both H and R enhanced caspase 9 activity after H/R compared to all other treatment groups.
Legend: Norm: Normoxia; Con: Control; ET: Endothelin-1; BOS: Bosentan; BE: BOS+ET; Con/BOS: treatment with BOS during reperfusion; ET/BOS: Hypoxia with ET and treatment with BOS during reperfusion; ET/ET: ET during hypoxia and reperfusion (H/R); BOS/BOS: BOS during H/R; BE/BE: BE during H/R.
Figure 61: Autophagy. H/R did not demonstrate increased MDC fluorescence. MDC fluorescence was increased only following Akt/PKB inhibition with FOS, with no effect seen after PKC inhibition with either Cal or Chel.

Legend: Con: Control; ET: Endothelin-1; BOS: Bosentan; Hypoxia and reperfusion (H/R); Cal: Calphostin C; Chel: Chelerythrine; FOS: Forskolin (PKA agonist).
Figure 62: Necrosis. H/R did not demonstrate increased PI fluorescence. No treatment group resulted in necrosis.

Legend: Con: Control; ET: Endothelin-1; BOS: Bosentan; Hypoxia and reperfusion (H/R); Cal: Calphostin C; Chel: Chelerythrine; FOS: Forskolin (PKA agonist).
Figure 63: NO production in HSVEC following H/R. H/R significantly impaired NO production. ET-1 exposure during hypoxia further reduced NO release. A significant reperfusion time dependent decrease in NO production was observed in all treatment groups. Exposure to the PKC inhibitors Cal and Chel significantly lowered NO release from HSVECs after H/R. However, co-incubation of ET-1 with PKC inhibitors demonstrated no added reduction in NO levels. HSVEC exposed to PMA demonstrated increased NO production compared to control and PMA significantly abrogated ET-1 induced effects. BOS treatment was effective at blocking ET-1 effects and increased NO production during reperfusion.

Legend: NO: Nitric Oxide; HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan.
Figure 64: NO production in HSVEC following H/R. PKA modulation did not alter H/R or ET-1 induced reductions in NO production. Akt/PKB inhibition significantly impaired NO production compared to Con. Co-incubation of SH5 with ET-1 further reduced NO release. Legend: NO: Nitric Oxide; HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; FOS: Forskolin (PKA agonist).
Figure 65: NO production in HSVEC following H/R. BOS treatment during reperfusion limited the effects of both H/R and ET-1. NO production demonstrated greater improvement after one hour of reperfusion compared to 24 hours. The exposure of ET-1 during reperfusion aggravated NO impairment compared to ET-1 during hypoxia alone.

Legend: NO: Nitric Oxide; HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; BOS: Bosentan; BE: BOS+ET; Con/BOS: treatment with BOS during reperfusion; ET/BOS: Hypoxia with ET and treatment with BOS during reperfusion; ET/ET: ET during hypoxia and reperfusion (H/R); BOS/BOS: BOS during H/R; BE/BE: BE during H/R.
Figure 66: NO production in HSVEC following H/R. ZI exposure reduced NO production following H/R in both Con and ET-1. DI exposure during hypoxia and reperfusion limited NO impairment in both Con and ET-1 exposed groups. EI therapy during hypoxia exacerbated NO impairment following H/R with further reduction observed when EI was given during reperfusion.

Legend: NO: Nitric oxide; HSVEC: Human saphenous vein endothelial cell; Con: Control; ET: Endothelin-1; ZI: PKC\(\lambda\) inhibitor, DI: Rottlerin; EI: PKC\(\varepsilon\) inhibitor.
Figure 67: ROS production and H/R. H/R increased ROS production compared to normoxia. ET-1 exposure during hypoxia enhanced ROS release. PKC inhibition resulted in significantly reduced ROS formation while PKC activation with PMA increased ROS production following H/R. Co-incubation of ET-1 with Cal, Chel, and PMA resulted in increased ROS production compared to Cal, Chel and PMA alone. PKA modulation did not alter H/R nor ET-1 induced ROS release. SH5 exposure increased ROS formation compared to H/R alone. BOS therapy was able to attenuate both H/R and ET-1 induced effects.

Legend: ROS: Reactive oxygen species; Con: Control; ET: Endothelin-1; BOS: Bosentan; Hypoxia and reperfusion (H/R); Cal: Calphostin C; Chel: Chelerythrine; FOS: Forskolin (PKA agonist).
Figure 68: ROS production and H/R. BOS treatment during reperfusion significantly reduced ROS formation. ET-1 exposure during reperfusion increased ROS production.

Legend: ROS: Reactive oxygen species; Con: Control; ET: Endothelin-1; BOS: Bosentan; BE: BOS+ET; Con/BOS: treatment with BOS during reperfusion; ET/BOS: Hypoxia with ET and treatment with BOS during reperfusion; ET/ET: ET during hypoxia and reperfusion (H/R); BOS/BOS: BOS during H/R; BE/BE: BE during H/R.
Figure 69: ROS production and H/R. Antioxidant therapy significantly reduces ROS production following H/R when given during hypoxia. Antioxidant therapy during both hypoxia and reperfusion further lowered ROS formation.

Legend: ROS: Reactive oxygen species; AO: Antioxidant; Con: Control; Sep: Sepiapterin (NOS cofactor); TI: Tiron (SOD anion); TE: Tempol (SOD mimetic).
Figure 70: ROS production and H/R. Antioxidant therapy significantly reduces ROS production following H/R when given during hypoxia in the ET-1 group. Antioxidant therapy during both hypoxia and reperfusion further lowered ROS formation after H/R in the ET-1 group.

Legend: ROS: Reactive oxygen species; AO: Antioxidant; ET: Endothelin-1; Sep: Sepiapterin (NOS cofactor); TI: Tiron (SOD anion); TE: Tempol (SOD mimetic).
Figure 71: ROS production and H/R. ZI and EI exposure during hypoxia resulted in significant ROS reduction after hypoxia and reperfusion while DI lowered free radical formation only during reperfusion. When isoform specific PKC inhibition was continued into the reperfusion phase a further reduction in ROS formation was observed. In the ET-1 exposed group all isoform specific inhibitors decreased ROS production.

Legend: ROS: Reactive oxygen species; No inh: No inhibitor; Con: Control; ET: Endothelin-1; ZI: PKC\(\lambda\) inhibitor; DI: Rottlerin; EI: PKC\(\epsilon\) inhibitor.
Figure 72: H/R and PKC activity. Hypoxia increases PKC activity. Reperfusion for both one and 24 hours increased PKC activity compared to normoxia and hypoxia. ET-1 treatment during hypoxia reduced consensus activity compared to hypoxia alone however the activity is higher than when ET-1 is given during normoxia. BOS treatment prevented the rise in PKC activity seen in the control group during H/R.

Legend: Con: Control; ET: Endothelin-1; BOS: Bosentan; H/R: hypoxia and reperfusion.
Figure 73: H/R and PKA activity. No differences were seen in PKA activity following H/R.
Legend: Con: Control; ET: Endothelin-1; BOS: Bosentan; H/R: Hypoxia and reperfusion.
Figure 74: Akt/PKB Western Blots. Significant differences were seen between groups following H/R. H/R significantly reduced serine phosphorylation compared to normoxia. When ET-1 was added during hypoxia a further reduction in serine phosphorylation was seen. H/R significantly reduced threonine phosphorylation compared to normoxia. When ET-1 was added during hypoxia a further reduction in threonine phosphorylation was seen. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist).
Figure 75: Akt/PKB Serine Phosphorylation. H/R significantly reduced serine phosphorylation compared to normoxia. When ET-1 was added during hypoxia a further reduction in serine phosphorylation was seen. BOS treatment during hypoxia limited the effect of hypoxia and one hour of reperfusion in both the control and ET-1 groups. Following 24 hours of reperfusion BOS abrogated ET-1 induced effects. PKC inhibition significantly reduced Akt/PKB serine phosphorylation following hypoxia and reperfusion. Co-incubation with ET-1 further lowered serine/total ratio during reperfusion but not after hypoxia alone. PMA decreased serine phosphorylation compared to control following 24 hours of reperfusion. An synergistic effect was seen with ET-1 co-incubation during one and 24 hours of reperfusion.

Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist).
Akt/PKB Phosphorylation

Figure 76: Akt/PKB Threonine Phosphorylation. H/R significantly reduced threonine phosphorylation compared to normoxia. When ET-1 was added during hypoxia a further reduction in threonine phosphorylation was seen. BOS treatment during hypoxia limited the effects of H/R and ET-1. At 24 hours of reperfusion the BOS groups demonstrated greater threonine phosphorylation. PKC inhibition significantly reduced Akt/PKB threonine phosphorylation following hypoxia and reperfusion. PMA exposure did not alter H/R or ET-1 induced effects.

Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist).
Figure 77: eNOS Western Blots. Significant differences were observed between groups following H/R. H/R significantly reduced eNOS expression following hypoxia compared to normoxia. ET-1 treatment during hypoxia further downregulated eNOS expression. BOS abrogated both hypoxia and ET-1 induced alterations in eNOS expression. During reperfusion eNOS expression increased and returned to normoxic levels after 24 hours. Akt/PKB and PKA modulation did not alter the effects of hypoxia or ET-1 on eNOS protein expression. PKC inhibition resulted in eNOS downregulation following hypoxia compared to control while PMA resulted in a slight upregulation. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist).
Figure 78: eNOS Expression. H/R significantly reduced eNOS expression following hypoxia compared to normoxia. ET-1 treatment during hypoxia further downregulated eNOS expression. BOS abrogated both hypoxia and ET-1 induced alterations in eNOS expression. During reperfusion eNOS expression increased and returned to normoxic levels after 24 hours. Akt/PKB and PKA modulation did not alter the effects of hypoxia or ET-1 on eNOS protein expression. PKC inhibition resulted in eNOS downregulation following hypoxia compared to control while PMA resulted in a slight upregulation. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist).
Figure 79: iNOS Western Blot images. No significant induction of iNOS was observed following H/R. No differences between groups were seen.
Figure 80: Caveolin-1 Western Blot images. No significant differences in caveolin-1 expression were observed.
Figure 81: Caveolin-1 Expression. No significant differences in caveolin-1 expression were observed.

Legend: ROS: Reactive oxygen species; Con: Control; ET: Endothelin-1; BOS: Bosentan; Hypoxia and reperfusion (H/R); Cal: Calphostin C; Chel: Chelerythrine; FOS: Forskolin (PKA antagonist).
Figure 82: PKCγ Western Blots. PKCγ translocation was significantly affected by H/R. Significant differences between groups were observed.

Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); H/R: hypoxia and reperfusion.
PKCδ Translocation

Figure 83: PKCδ translocation is significantly increased following H/R. ET-1 exposure further translocated PKCδ to the membrane. PKC inhibition attenuated the effects of H/R on delta translocation. PKA and PKB modulation did not alter H/R and ET-1 induced changes in PKCδ translocation.

Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); H/R: Hypoxia and reperfusion.
Figure 84: PKCε Western Blots. PKCε translocation was significantly affected by H/R. Significant differences between groups were observed.

Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); H/R: Hypoxia and reperfusion.
Figure 85: PKCε Translocation. PKCε translocation is significantly increased following H/R. ET-1 exposure further translocated PKCε to the membrane. PKA and PKB modulation did not alter H/R and ET-1 induced changes in PKCε translocation. Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); H/R: Hypoxia and reperfusion.
Figure 86: PKC\(\lambda\) Western Blots. PKC\(\lambda\) translocation was significantly affected by H/R. Significant differences between groups were observed.

Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); H/R: Hypoxia and reperfusion.
PKCλ Translocation

Figure 87: PKCλ translocation is significantly reduced following H/R. ET-1 exposure further reduced the PKCλ M/C ratio. Neither PKA nor Akt/PKB modulation altered H/R and ET-1 induced changes in PKCλ translocation.

Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist); H/R: Hypoxia and reperfusion.
Figure 88: eNOS Western Blots. Images depict eNOS cytosolic and membrane expression. Significant differences were found between groups.
Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA antagonist); H/R: Hypoxia and reperfusion.
Figure 89: eNOS Localization. Hypoxia significantly increased the eNOS M/C ratio. ET-1 treatment during hypoxia further localized eNOS to the membrane after hypoxia. BOS therapy limited the effects of hypoxia on eNOS translocation. Akt/PKB inhibition during hypoxia demonstrated a higher M/C ratio compared to control with an additive increase in eNOS localization when co-incubated with ET-1. PKC inhibition during hypoxia increased the M/C ratio with a further increase with ET-1 co-incubation. PMA exposure revealed eNOS localization to the membrane following hypoxia and a greater membrane presence of eNOS when co-incubated with ET-1. All groups demonstrated a return to a normoxic M/C ratio following one or 24 hours of reperfusion.

Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel Chelerythrine; BOS: Bosentan; FOS: Forskolin (PKA agonist).
Figure 90: Endothelial dependent vasodilation in rat thoracic aorta. The graph depicts the cumulative dose-response curves to Ach in aortic segments. CyA treatment resulted in impaired endothelial dependent vasorelaxation compared to SRL and Con. SRL exposure improved endothelial dependent vasorelaxation compared to control.

Legend: Ach: Acetylcholine; CyA: Cyclosporine; SRL: Rapamycin; Con: Control.
Figure 91: Endothelial dependent vasodilation in rat thoracic aorta. The graph depicts the cumulative dose-response curves to Ach in aortic segments. BH4 treatment limited CyA induced endothelial dysfunction but failed to restore the vasculature to normal Con function.

Legend: Ach: Acetylcholine; CyA: Cyclosporine; Con: Control; BH4: Tetrahydrobiopterin.
Figure 92: Endothelial dependent vasodilation in rat thoracic aorta. The graph depicts the cumulative dose-response curves to Ach in aortic segments. BOS treatment completely abrogated CyA induced endothelial dysfunction. No significant differences were seen between Con and BOS treated groups.

Legend: Ach: Acetylcholine; CyA: Cyclosporine; Con: Control; BOS: Bosentan.
Figure 93: Endothelial dependent vasodilation in rat thoracic aorta. The graph depicts the cumulative dose-response curves to Ach in aortic segments. Combined BOS and BH₄ treatment completely abrogated CyA induced endothelial dysfunction. Combined treatment increased vasodilatory response to Ach compared to Con.

Legend: Ach: Acetylcholine; CyA: Cyclosporine; Con: Control; BOS: Bosentan; BH₄: Tetrahydrobiopterin.
Figure 94: Endothelial dependent vasodilation in rat thoracic aorta. The graph depicts the endothelial dependent vasorelaxation at maximal Ach concentration. CyA treatment resulted in the lowest Emax% compared to all experimental groups. Combined BOS and BH4 groups had the highest Emax% compared to Con and all other treatment groups.

Legend: Ach: Acetylcholine; CyA: Cyclosporine; Con: Control; SRL: Rapamycin; BOS: Bosentan; BH4: Tetrahydrobiopterin.
Figure 95: Endothelial dependent vasodilation in rat thoracic aorta. The graph depicts the concentration of Ach required to elicit half maximal vasodilation (ED\textsubscript{50}). CyA treatment resulted in the highest ED\textsubscript{50} compared to all experimental groups. BH\textsubscript{4} attenuated the CyA-induced increase in the Ach ED\textsubscript{50}. BOS and the combined BOS and BH\textsubscript{4} groups had lower ED\textsubscript{50} compared to Con. SRL exposure resulted in the lowest ED\textsubscript{50} compared to all treated groups.

Legend: Ach: Acetylcholine; CyA: Cyclosporine; Con: Control; SRL: Rapamycin; BOS: Bosentan; BH\textsubscript{4}: Tetrahydrobiopterin.
Figure 96: Endothelial independent vasodilation in rat thoracic aorta. The graph depicts the cumulative dose-response curves to SNP in aortic segments. CyA treatment resulted in impaired endothelial independent vasorelaxation compared to SRL and Con.

Legend: SNP: sodium nitroprusside; CyA: Cyclosporine; Con: Control; SRL: Rapamycin.
Figure 97: Endothelial independent vasorelaxation in rat thoracic aorta. The graph depicts the cumulative dose-response curves to SNP in aortic segments. BH₄ treatment limits CyA induced dysfunction.

Legend: SNP: sodium nitroprusside; CyA: Cyclosporine; Con: Control; BH₄: Tetrahydrobiopterin.
Figure 98: Endothelial independent vasodilation in rat thoracic aorta. The graph depicts the cumulative dose-response curves to SNP in aortic segments. BOS treatment abrogates CyA induced dysfunction.

Legend: SNP: sodium nitroprusside; CyA: Cyclosporine; Con: Control; BOS: Bosentan.
Figure 99: Endothelial independent vasodilation in rat thoracic aorta. The graph depicts the cumulative dose-response curves to SNP in aortic segments. Combination therapy with or without CyA resulted in improved vasodilation to SNP than control. Legend: SNP: sodium nitroprusside; CyA: Cyclosporine; Con: Control; BOS: Bosentan; BH4: Tetrahydrobiopterin.
Figure 100: Endothelial independent vasodilation in rat thoracic aorta. The graph depicts the endothelial independent vasorelaxation at maximal SNP concentration. No significant differences were seen between Con and CyA groups. The combined BOS and BH₄ group as well as the BH₄ alone group had the highest Emax% compared to Con and all other treatment groups.

Legend: SNP: sodium nitroprusside; CyA: Cyclosporine; Con: Control; BOS: Bosentan; BH₄: Tetrahydrobiopterin.
Figure 101: Endothelial independent vasodilation in rat thoracic aorta. The graph depicts the concentration of SNP required to elicit half maximal vasodilation (ED\textsubscript{50}). CyA treatment resulted in the highest ED\textsubscript{50} compared to all experimental groups. BH\textsubscript{4} attenuated the CyA-induced increase in the SNP ED\textsubscript{50}. The combined BOS and BH\textsubscript{4} group had the lowest ED\textsubscript{50} compared to all groups.

Legend: SNP: sodium nitroprusside; CyA: Cyclosporine; Con: Control; SRL: Rapamycin; BOS: Bosentan; BH\textsubscript{4}: Tetrahydrobiopterin.
Figure 102: Sensitivity to Vasospasm. Cumulative dose-response curves to ET-1 in aortic segments. CyA increased vasosensitivity to ET-1 compared to Con and SRL. Legend: ET-1: Endothelin-1; CyA: Cyclosporine; Con: Control; SRL: Rapamycin.
Figure 103: Sensitivity to Vasospasm. Cumulative dose-response curves to ET-1 in aortic segments. All three treatment groups (CyA, BH4+CyA, and BH4) demonstrated increased vasosensitivity to ET-1 compared to Con. However, no significant differences were seen between CyA, BH4+CyA, and BH4 exposed rats.

Legend: ET-1: Endothelin-1; CyA: Cyclosporine; Con: Control; BH4: Tetrahydrobiopterin.
Figure 104: Sensitivity to Vasospasm. Cumulative dose-response curves to ET-1 in aortic segments. BOS treatment (with or without CyA) resulted in a reduced sensitivity to ET-1 induced vasospasm compared to Con.

Legend: ET-1: Endothelin-1; CyA: Cyclosporine; Con: Control; BOS: Bosentan.
Figure 105: Sensitivity to Vasospasm. Cumulative dose-response curves to ET-1 in aortic segments. Combined BOS and BH4 treatment (with or without CyA) resulted in a reduced sensitivity to ET-1 induced vasospasm compared to Con.

Legend: ET-1: Endothelin-1; CyA: Cyclosporine; Con: Control; BH4: Tetrahydrobiopterin; BOS: Bosentan.

* p<0.05 vs. Con

Group*dose effect p<0.0001
Figure 106: The graph depicts vasoreactivity at maximal ET-1 concentrations. CyA treatment resulted in a greater %Cmax compared to Con. The combined BOS and BH4 group had the lowest %Cmax compared to Con and all other treatment groups. BH4 exposure (with or without CyA) resulted in increased vasospasm.

Legend: ET-1: Endothelin-1; CyA: Cyclosporine; Con: Control; BH4: Tetrahydrobiopterin; BOS: Bosentan.
Figure 10: The graph depicts the concentration of ET-1 required to elicit half maximal vasocontraction (ED$_{50}$). CyA treatment resulted in a reduced ED$_{50}$ compared to Con. BH$_4$ exposure also lowered the ED$_{50}$ compared to Con. BOS and the combined BOS and BH$_4$ groups increased the ED$_{50}$ compared to all experimental groups.

Legend: ET-1: Endothelin-1; CyA: Cyclosporine; BH$_4$: Tetrahydrobiopterin; BOS: Bosentan.
Figure 108: Plasma ET-1 Levels. CyA exposure did not alter plasma ET-1 levels compared to control. SRL treated animals demonstrated a significantly lower plasma ET-1 level. BH4 and BH4+CyA treatment resulted in reduced ET-1 plasma levels compared to both control and CyA treated animals. BOS exposure also reduced plasma ET-1 levels.

Legend: ET-1: Endothelin-1; CyA: Cyclosporine; Con: Control; BH4: Tetrahydrobiopterin; BOS: Bosentan.
8-Isoprostane Levels

Group p<0.0001
* p<0.05 vs. Con
# p<0.05 vs. CyA
& p<0.05 vs. all groups

Con CyA SRL BH$_4$CyA BH$_4$ BOSCyA BOS BBCyA BB

Figure 109: This figure displays 8-isoprostane levels as a percent change from baseline. CyA increased ROS injury compared to all groups. BOS therapy significantly abrogated CyA-induced oxidative injury. BH$_4$ completely attenuated CyA free radical injury while BH$_4$ alone reduced ROS injury to below control levels.
Legend: ROS: Reactive oxygen species; Con: Control; CyA: Cyclosporine; SRL: Rapamycin; BOS: Bosentan; BH$_4$: Tetrahydrobiopterin; BB: BOS+BH$_4$. 
Figure 110: This figure demonstrates ROS production as a percentage of control in human saphenous vein endothelial cells. A. CyA increased ROS production compared to all groups. BOS therapy significantly abrogated CyA oxidative burst. BOS exposure lowered free radical production compared to Con. B. BH₄ partially attenuated CyA induced ROS production while BH₄ alone reduced free radical release to below control levels.

Legend: ET-1: Endothelin-1; CyA: Cyclosporine; Con: Control; BH₄: Tetrahydrobiopterin; BOS: Bosentan.
Figure 111: A. Western Blot for ETβ Receptor expression demonstrated upregulation following CyA treatment. B. Quantitative Western blot analysis of ETβ Receptor expression in the thoracic aorta. CyA treatment increased ETβ Receptor expression compared to SRL and Con. BH4 and BOS therapy abrogated CyA-induced upregulation.

Legend: Rc: Receptor; Con: Control, Cyclosporine: CyA; SRL: Rapamycin, BH4: Tetrahydrobiopterin, BOS: Bosentan, BB: BOS+BH4.
Figure 112: A. Western Blot for ETβ receptor Rc expression demonstrated upregulation following BH4 treatment. B. Quantitative Western blot analysis of ETβ Rc expression in the thoracic aorta. BH4 treatment increased ETβ Rc expression compared to all treatment groups. BOS therapy abrogated BH4-induced upregulation.

Legend: Rc: Receptor; Con: Control, Cyclosporine: CyA; SRL: Rapamycin, BH4: Tetrahydrobiopterin, BOS: Bosentan, BB: BOS+BH4.
Figure 113: A. i-iv) Western Blot for eNOS protein expression demonstrated downregulation following CyA treatment. BH4 and BOS exposure limits CyA-induced downregulation while combined therapy restores normal eNOS expression. v) Western Blot for iNOS protein expression demonstrating no differences between groups. B. Quantitative Western blot analysis of eNOS expression in the thoracic aorta.

Figure 114: A. Western Blot for TNF-α protein expression demonstrated no significant differences between groups. B. Quantitative Western blot analysis of TNF-α expression in the left ventricle revealed no differences between groups.

Figure 115: A. Western Blot for TGF-β protein expression demonstrated no significant differences between groups. B. Quantitative Western blot analysis of TGF-β expression in the left ventricle revealed no differences between groups.

## 8.2 TABLES

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<tr>
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<td>Proximal coronary vessels.</td>
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<tr>
<td><strong>Plaque pattern</strong></td>
<td>Diffuse &amp; Concentric</td>
<td>Focal &amp; Eccentric</td>
</tr>
<tr>
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<td>Yes</td>
<td>Rarely</td>
</tr>
<tr>
<td><strong>Internal elastic lamina</strong></td>
<td>Intact</td>
<td>Disrupted</td>
</tr>
<tr>
<td><strong>Calcium deposition</strong></td>
<td>No</td>
<td>Yes</td>
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Table 1: Comparison between CAV and CAD.
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<tr>
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<th>BH₄</th>
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<th>BOS</th>
<th>BOS+ BH₄+CyA</th>
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<td></td>
<td></td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>%Cmax</td>
<td>↑↑</td>
<td>±</td>
<td>↑↑↑</td>
<td>↑↑</td>
<td>↓↓</td>
<td>↓↓</td>
<td>↓↓</td>
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<td>±</td>
<td>↓↓</td>
<td>↓↓</td>
<td>↓↓</td>
<td>↓↓</td>
<td>↓↓</td>
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<td>±</td>
<td>±</td>
<td>↓</td>
<td>±</td>
<td>±</td>
<td>±</td>
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<td>±</td>
<td>±</td>
<td>±</td>
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<td>±</td>
<td>±</td>
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<td>±</td>
<td>±</td>
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<td>↓</td>
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</tr>
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<td>±</td>
<td>±</td>
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<td>±</td>
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<td></td>
<td></td>
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<tr>
<td>Emax%</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
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<tr>
<td>eNOS</td>
<td>↓↓</td>
<td>±</td>
<td>↓</td>
<td>±</td>
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<td>±</td>
<td>↓</td>
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Table 2: Summary of vascular effects. CyA: Cyclosporine; SRL: Rapamycin; BH₄: Tetrahydrobiopterin; ET-1: Endothelin-1; Ach: Acetylcholine; SNP: sodium nitroprusside; ROS: Reactive Oxygen Species; eNOS: endothelial nitric oxide synthase; ET-1Rc: ET-1 receptor (either A or B) Emax%: % maximal relaxation, %Cmax: % maximal contraction.
CHAPTER NINE

APPENDIX
Appendix 1:

<table>
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<th>Group</th>
<th>Total Nitrite Levels (µM/mg)</th>
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<tbody>
<tr>
<td>Con</td>
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<td>ET 100pM</td>
<td>#7±0.3</td>
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<tr>
<td>ET 1nM</td>
<td>*10±1.0</td>
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<tr>
<td>ET 10nM</td>
<td>*5±0.3</td>
</tr>
<tr>
<td>ET 100nM</td>
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PKC\(\lambda\) Activity Summary Data.
Legend: Con: Control; ET: Endothelin-1;
* p<0.05 vs. Con, # p<0.05 vs. ET 100nM,
Appendix 2:

<table>
<thead>
<tr>
<th>Group</th>
<th>Time</th>
<th>Levels (µM/mg)</th>
<th>Group</th>
<th>Time</th>
<th>Levels (µM/mg)</th>
<th>Group</th>
<th>Time</th>
<th>Levels (µM/mg)</th>
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<td>BOS</td>
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<td>@&amp;*7.3±0.1</td>
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<tr>
<td>ET</td>
<td>30 Min</td>
<td>#*5.2±0.1</td>
<td>BOS+ET</td>
<td>30 Min</td>
<td>@&amp;*6.5±0.6</td>
<td>24 Hour</td>
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<tr>
<td>Cal</td>
<td>30 Min</td>
<td>@&amp;*2.2±0.4</td>
<td>SH5</td>
<td>30 Min</td>
<td>@*4.8±0.3</td>
<td>24 Hour</td>
<td>@&amp;*1.3±0.2</td>
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<td>Cal+ET</td>
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<td>SH5+ET</td>
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<td>@&amp;*1.1±0.2</td>
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<td>Chel</td>
<td>30 Min</td>
<td>@&amp;*1.4±0.1</td>
<td>FOS</td>
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<td>@&amp;*6.6±1.2</td>
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<td>@&amp;*1.5±0.2</td>
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<td>Chel+ET</td>
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<td>@&amp;*1.4±0.3</td>
<td>FOS+ET</td>
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<td>@&amp;*5.7±0.3</td>
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<td>@&amp;*1.2±0.5</td>
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<tr>
<td>PMA</td>
<td>30 Min</td>
<td>@&amp;*8.4±0.8</td>
<td>H89</td>
<td>30 Min</td>
<td>@&amp;*6.3±0.3</td>
<td>24 Hour</td>
<td>@&amp;*11.9±0.4</td>
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</tr>
<tr>
<td>PMA+ET</td>
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<td>H89+ET</td>
<td>30 Min</td>
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<td>24 Hour</td>
<td>@&amp;*11.8±0.1</td>
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</table>

Total Nitrite Summary Data.
Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin; ZI: PKCε inhibitor; DI: Rottlerin; EI: PKCε inhibitor.
* p<0.05 vs. Con 30 min, # p<0.05 vs. Con 24 hr, & p<0.05 vs. ET 30min, @ p<0.05 vs. ET 24hr
Appendix 3:

<table>
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<tr>
<th>Group</th>
<th>ROS Production</th>
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<th>ROS Production</th>
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<td>*79±5</td>
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<td>#92±5</td>
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<td>DI+ET</td>
<td>#98±4</td>
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<td>E1</td>
<td>#*79±3</td>
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<td>Cal+ET</td>
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<td>Chel</td>
<td>*80±2</td>
<td>Sep</td>
<td>94±6</td>
</tr>
<tr>
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<td>*96±3</td>
<td>Sep+ET</td>
<td>*#114±3</td>
</tr>
<tr>
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<td>*114±8</td>
<td>L-NAME</td>
<td>#92±4</td>
</tr>
<tr>
<td>PMA+ET</td>
<td>*#143±2</td>
<td>L-NAME+ET</td>
<td>#112±2</td>
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<td>SH5</td>
<td>*116±5</td>
<td>Apocynin</td>
<td>96±7</td>
</tr>
<tr>
<td>SH5+ET</td>
<td>*#164±2</td>
<td>Apocynin+ET</td>
<td>*#120±5</td>
</tr>
<tr>
<td>FOS</td>
<td>93±8</td>
<td>TI</td>
<td>92±5</td>
</tr>
<tr>
<td>FOS+ET</td>
<td>*126±3</td>
<td>TI+ET</td>
<td>*135±8</td>
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<td>H89</td>
<td>95±4</td>
<td>TE</td>
<td>*#87±3</td>
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<td>*122±3</td>
<td>TE+ET</td>
<td>*#127±4</td>
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ET value for Analysis with antioxidant was 139±2

ROS Summary Data.
Legend: ROS: Reactive Oxygen Species; Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin; ZI: PKCλ inhibitor; DI: Rottlerin; EI: PKCε inhibitor.

* p<0.05 vs. Con, # p<0.05 vs. ET

= p<0.05
### Appendix 4:

<table>
<thead>
<tr>
<th>PKCδ Translocation</th>
<th>PKCε Translocation</th>
<th>PKCλ Translocation</th>
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<td>M/C Ratio</td>
<td>M/C Ratio</td>
<td>M/C Ratio</td>
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<td>24 hours</td>
<td>30 minutes</td>
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<tr>
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<td>@#1.2±0.1</td>
<td>@#1.2±0.1</td>
</tr>
<tr>
<td>ET</td>
<td>@*2.3±0.1</td>
<td>@&amp;*3.1±0.1</td>
</tr>
<tr>
<td>Cal</td>
<td>@&amp;*0.8±0.2</td>
<td>@&amp;#*0.8±0.2</td>
</tr>
<tr>
<td>Cal+ET</td>
<td>@&amp;*0.5±0.1</td>
<td>@&amp;#*0.6±0.1</td>
</tr>
<tr>
<td>Chel</td>
<td>@*0.7±0.1</td>
<td>@&amp;*0.7±0.1</td>
</tr>
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<td>@&amp;*0.5±0.2</td>
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<td>@*2.5±0.2</td>
<td>@&amp;*3.1±0.2</td>
</tr>
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<td>@#1.2±0.1</td>
<td>@#1.1±0.1</td>
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<td>BOS+ET</td>
<td>@#1.1±0.1</td>
<td>@#1.1±0.1</td>
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<td>SH5</td>
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<tr>
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<td>@#1.3±0.1</td>
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<td>@#1.3±0.2</td>
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<td>@*2.3±0.3</td>
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PKC Translocation Summary Data.

Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin.

* p<0.05 vs. Con 30 min, # p<0.05 vs. ET 30 min, & p<0.05 vs. Con 24 hr, @ p<0.05 vs. ET 24hr.
Appendix 5:

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<th>PKC Activity (U/mg)</th>
<th>PKA Activity (U/mg)</th>
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<td>*2.1±0.4</td>
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<td>&amp;#0.7±0.1</td>
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<td>&amp;#0.5±0.1</td>
</tr>
<tr>
<td>Chel</td>
<td>*1.3±0.1</td>
<td>*0.7±0.2</td>
</tr>
<tr>
<td>Chel+ET</td>
<td>*1.2±0.2</td>
<td>*0.7±0.2</td>
</tr>
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<td>*6.4±0.2</td>
<td>*9.4±0.7</td>
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<td>PMA+ET</td>
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<td>*9.0±0.3</td>
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<td>BOS</td>
<td>*4.3±0.1</td>
<td>*5.3±0.5</td>
</tr>
<tr>
<td>BOS+ET</td>
<td>*4.0±0.2</td>
<td>*5.4±0.2</td>
</tr>
<tr>
<td>SH5</td>
<td>*3.4±0.1</td>
<td>*3.4±0.1</td>
</tr>
<tr>
<td>SH5+ET</td>
<td>*3.2±0.03</td>
<td>*2.2±0.4</td>
</tr>
<tr>
<td>FOS</td>
<td>*3.7±0.3</td>
<td>*3.9±0.4</td>
</tr>
<tr>
<td>FOS+ET</td>
<td>*3.1±0.1</td>
<td>*2.1±0.4</td>
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<tr>
<td>H89</td>
<td>*3.7±0.4</td>
<td>*3.8±0.4</td>
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<tr>
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PKC and PKA Activity Summary Data. Values expressed as arbitrary units per mg of protein
Legend: PKC: Protein kinase C; PKA: Protein kinase A; Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin.
PKC: * p<0.05 vs. Con 30 min, # p<0.05 vs. ET 30 min, & p<0.05 vs. ET 24 hr.
PKA: @ p<0.05 vs Con 30 min, ^ p<0.05 vs. Con 24 hr.
Appendix 6:

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<tr>
<td>-ve Con</td>
<td>9±5%</td>
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</table>

PKC\(\lambda\) Activity Summary Data.
Legend: Con: Control; ZI: PKC\(\lambda\) inhibitor; ET: Endothelin-1;
* p<0.05 vs. Con, # p<0.05 vs. ET, & p<0.05 vs. ZI,
Appendix 7:

<table>
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<th>Ser/Total Ratio</th>
<th>Thr/Total Ratio</th>
<th>Ser/Total Ratio</th>
<th>Thr/Total Ratio</th>
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<td>24 hours</td>
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<td>0.7±0.10</td>
<td>&amp;1.4±0.09</td>
<td>0.7±0.07</td>
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<td>#1.9±0.09</td>
<td>0.8±0.10</td>
<td>@2.0±0.10</td>
<td>0.7±0.04</td>
</tr>
<tr>
<td>BOS+ET</td>
<td>#2.0±0.10</td>
<td>0.8±0.08</td>
<td>@2.0±0.10</td>
<td>0.6±0.07</td>
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Akt/PKB Phosphorylation Summary Data.
Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan;
* p<0.05 vs. Con 30 min, # p<0.05 vs. ET 30 min, & p<0.05 vs. Con 24 hr,
@ p<0.05 vs. ET 24hr.

= p<0.05
Appendix 8:

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eNOS Protein Expression Summary Data.
Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin. * p<0.05 vs. Con 30 min, # p<0.05 vs. ET 30 min, & p<0.05 vs. Con 24 hr, @ p<0.05 vs. ET 24hr. ○○○ = p<0.05
### Appendix 9: Cell Viability Summary Data

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Cell Viability Summary Data.
Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin; Con/BOS: treatment with BOS during reperfusion; ET/BOS: Hypoxia with ET and treatment with BOS during reperfusion; ET/ET: ET during hypoxia and reperfusion (H/R); BOS/BOS: BOS during H/R.
*p<0.05 vs. Norm 4hr, # p<0.05 vs. Norm 24hr, & p<0.05 vs. ET 4hr, @ p<0.05 vs. ET 24hr
### Homogeneous Caspase Activity

(% increase fluorescence (%))

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Homogeneous Caspase Activity Summary Data.
Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin; H2O2: Hydrogen peroxide; Con/BOS: treatment with BOS during reperfusion; ET/BOS: Hypoxia with ET and treatment with BOS during reperfusion; ET/ET: ET during hypoxia and reperfusion (H/R); BOS/BOS: BOS during H/R.

* p<0.05 vs. Norm, @ p<0.05 vs. Con 0hr, # p<0.05 vs. ET 0hr
Appendix 11:

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Homogeneous Caspase Activity And PKC inhibitors Summary Data.
Legend: Con (0hr): Control no reperfusion; Con (1hr): Control one hour reperfusion; Con (24hr) Control 24 hour reperfusion; ET (0hr): Endothelin-1 no reperfusion; ET (1hr): Endothelin-1 one hour reperfusion; ET (24hr) Endothelin-1 24 hour reperfusion; No inh: No inhibitor; ZI: PKCα inhibitor; DI: Rottlerin; EI: PKCε Inhibitor.
* p<0.05 vs. Con (0hr), # p<0.05 vs. ET (0hr).
Appendix 12:

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Homogeneous Caspase Activity Summary Data.
Legend: Con (0hr): Control no reperfusion; Con (1hr): Control one hour reperfusion; Con (24hr) Control 24 hour reperfusion; ET (0hr): Endothelin-1 no reperfusion; ET (1hr): Endothelin-1 one hour reperfusion; ET (24hr) Endothelin-1 24 hour reperfusion; No inh: No inhibitor; AO: Antioxidant; Sep: Sepiapterin; TE: Tempol; TI: Tiron.
* p<0.05 vs. Con (0hr), # p<0.05 vs. ET (0hr).
Appendix 13:

<table>
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Homogeneous Caspase Activity Summary Data.
Legend: BOS (0hr): Bosentan no reperfusion; BOS (1hr): Bosentan one hour reperfusion; BOS (24hr) Bosentan 24 hour reperfusion; BOS+ET (0hr): Bosentan+Endothelin-1 no reperfusion; BOS+ET (1hr): Bosentan+Endothelin-1 one hour reperfusion; BOS+ET (24hr) Bosentan+Endothelin-1 24 hour reperfusion; No inh: No inhibitor; AO: Antioxidant; Sep: Sepiapterin; TE: Tempol; TI: Tiron.

* p<0.05 vs. BOS (0hr), # p<0.05 vs. BOS+ET (0hr).
Figure: Caspase 8 Fluorescence. Caspase 8 fluorescence after hypoxia and 24 hours of reperfusion.
Legend: Con: Control; ET: Endothelin-1; BOS: Bosentan.
Appendix 15:

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Homogeneous Caspase Activity Summary Data.
Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin; Con/BOS: treatment with BOS during reperfusion; ET/BOS: Hypoxia with ET and treatment with BOS during reperfusion; ET/ET: ET during hypoxia and reperfusion (H/R); BOS/BOS: BOS during H/R.
* p<0.05 vs. Norm, @ p<0.05 vs. Con 0hr.
Figure: Caspase 9 Fluorescence. Caspase 9 fluorescence after hypoxia and 24 hours of reperfusion.
Legend: Con: Control; ET: Endothelin-1; BOS: Bosentan.
Appendix 17:

Homogeneous Caspase Activity Summary Data.

Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin; Con/BOS: treatment with BOS during reperfusion; ET/BOS: Hypoxia with ET and treatment with BOS during reperfusion; ET/ET: ET during hypoxia and reperfusion (H/R); BOS/BOS: BOS during H/R.

* p<0.05 vs. Norm, @ p<0.05 vs. Con 0hr.

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Autophagy Summary Data.
Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin.
* p<0.05 vs. SH5 (Norm)
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PI Fluorescence Summary Data.
Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin.
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<th>24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>@7.29±0.14</td>
<td>*4.08±0.24</td>
<td>*3.77±0.34</td>
<td>*4.07±0.14</td>
</tr>
<tr>
<td>ET</td>
<td>*4.27±0.18</td>
<td>@*2.89±0.28</td>
<td>@*2.71±0.38</td>
<td>@*2.33±0.18</td>
</tr>
<tr>
<td>BOS</td>
<td>@*9.03±0.41</td>
<td>@*5.14±0.31</td>
<td>@7.86±0.21</td>
<td>@6.75±0.41</td>
</tr>
<tr>
<td>BOS+ET</td>
<td>@*8.79±0.36</td>
<td>@*5.16±0.26</td>
<td>@7.51±0.16</td>
<td>@6.16±0.36</td>
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<tr>
<td>Cal</td>
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<td>@*0.84±0.18</td>
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<td>@*0.56±0.15</td>
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<td>@*0.85±0.19</td>
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</tr>
<tr>
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<td>@*0.72±0.15</td>
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<tr>
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<tr>
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<td>7.22±0.22</td>
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<tr>
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<td>@*1.36±0.17</td>
<td>@*0.91±0.15</td>
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<tr>
<td>BOS+ET/BOS+ET</td>
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<td>@*0.66±0.17</td>
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NO production Following H/R Summary Data.

Legend: NO: Nitric Oxide; Con: Control; ET: Endothelin-1; Norm: Normoxia; 0hr: no reperfusion; 1hr: one hour reperfusion; 24hr: 24 hour reperfusion; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin; Con/BOS: treatment with BOS during reperfusion; ET/BOS: Hypoxia with ET and treatment with BOS during reperfusion; ET/ET: ET during hypoxia and reperfusion (H/R); BOS/BOS: BOS during H/R.

* p<0.05 vs. Con Norm, @ p<0.05 vs. Con 0hr
Appendix 21:

<table>
<thead>
<tr>
<th>Group</th>
<th>Hyp</th>
<th>H/R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con (0hr)</td>
<td>No inh</td>
<td>#4.08±0.24</td>
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<tr>
<td></td>
<td>ZI</td>
<td>*1.04±0.12</td>
</tr>
<tr>
<td></td>
<td>DI</td>
<td>#3.75±0.10</td>
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<td>EI</td>
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<tr>
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<td>DI</td>
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<tr>
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<td>ZI</td>
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<tr>
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<td>DI</td>
<td>#4.38±0.23</td>
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<td>EI</td>
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<tr>
<td>ET (0hr)</td>
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<tr>
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<td>ZI</td>
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<td>DI</td>
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<td>EI</td>
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<tr>
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<td>EI</td>
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</table>

NO production Following H/R Summary Data.
Legend: NO: Nitric Oxide; Con: Control; ET: Endothelin-1; Norm: Normoxia; 0hr: no reperfusion; 1hr: one hour reperfusion; 24hr: 24 hour reperfusion; No inh: No inhibitor; ZI: PKCλ inhibitor; DI: Rottlerin; EI: PKCε Inhibitor.

* p<0.05 vs. Con (0hr) No inh, # p<0.05 vs. ET (0hr) No inh.
Appendix 22:

<table>
<thead>
<tr>
<th>Group</th>
<th>Norm</th>
<th>0 hr</th>
<th>1 hr</th>
<th>24 hr</th>
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<tbody>
<tr>
<td>Con</td>
<td>100</td>
<td>110±3</td>
<td>*154±3</td>
<td>*211±7</td>
</tr>
<tr>
<td>ET</td>
<td>*130±3</td>
<td>*183±5</td>
<td>*258±2</td>
<td>*352±3</td>
</tr>
<tr>
<td>BOS</td>
<td>*80±5</td>
<td>*72±2</td>
<td>*67±5</td>
<td>*154±6</td>
</tr>
<tr>
<td>BOS+ET</td>
<td>*77±2</td>
<td>*78±7</td>
<td>*79±8</td>
<td>*169±5</td>
</tr>
<tr>
<td>Cal</td>
<td>*73±3</td>
<td>*91±3</td>
<td>*146±3</td>
<td>*212±8</td>
</tr>
<tr>
<td>Cal+ET</td>
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<td>*124±8</td>
<td>*194±6</td>
<td>*254±3</td>
</tr>
<tr>
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<td>*74±2</td>
<td>*97±5</td>
<td>*199±6</td>
</tr>
<tr>
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<td>*125±6</td>
<td>*149±6</td>
<td>*249±8</td>
</tr>
<tr>
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<td>*140±5</td>
<td>*181±8</td>
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<tr>
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<td>*122±7</td>
<td>*171±5</td>
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<tr>
<td>ET/BOS</td>
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<tr>
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<td>*78±7</td>
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<td>ET/BE</td>
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<td>SH5+ET/BOS</td>
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<td>*236±7</td>
<td>*254±9</td>
<td>*307±11</td>
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</table>

ROS production Following H/R Summary Data.
Legend: ROS: Reactive oxygen species; Con: Control; ET: Endothelin-1; Norm: Normoxia; 0hr: no reperfusion; 1hr: one hour reperfusion; 24hr: 24 hour reperfusion; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin; Con/BOS: treatment with BOS during reperfusion; ET/BOS: Hypoxia with ET and treatment with BOS during reperfusion; ET/ET: ET during hypoxia and reperfusion (H/R); BOS/BOS: BOS during H/R.
* p<0.05 vs. Con Norm, @ p<0.05 vs. ET Norm
Appendix 23:

ROS Production Following H/R Summary Data.

Legend: ROS: Reactive oxygen species; AO: Antioxidant; Con: Control; ET: Endothelin-1; Sep: Sepiapterin; TI: Tiron; TE: Tempol.

* p<0.05 vs. Con No AO, @ p<0.05 vs. ET No AO

<table>
<thead>
<tr>
<th>Group (0hr)</th>
<th>Hyp</th>
<th>H/R</th>
<th>Group (1hr)</th>
<th>Hyp</th>
<th>H/R</th>
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<td>No AO</td>
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<td>Sep</td>
<td>@*139±7</td>
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<td>@*92±7</td>
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<td>@*161±4</td>
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<td>TI</td>
<td>@*165±3</td>
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</table>

| Con (1hr)  | No AO | @*154±3 | @*154±4 | ET (1hr) | No AO | @*258±2 | @*258±3 |
| Sep        | @106±4 | @*96±2 | Sep        | @*161±5 | @*145±6 |
| L-NAME     | @101±5 | @*90±4 | L-NAME     | @*167±3 | @*148±8 |
| Apocynin   | @113±3 | @103±3 | Apocynin   | *182±3 | @*161±7 |
| TE         | @*128±6 | @*93±7 | TE         | *193±4 | @*170±9 |
| TI         | @*136±5 | @*92±2 | TI         | @*199±3 | @*173±8 |

| Con (24hr) | No AO | @*211±7 | @*211±7 | ET (24hr) | No AO | @*352±3 | @*352±3 |
| Sep        | @*131±2 | @105±3 | Sep        | @*200±10 | @170±7 |
| L-NAME     | @*123±6 | @107±4 | L-NAME     | @*192±9 | @*177±11 |
| Apocynin   | *189±7 | @*135±5 | Apocynin   | @*215±5 | @*199±6 |
| TE         | @*201±6 | @116±9 | TE         | @*251±8 | @*203±7 |
| TI         | @*216±8 | @*122±7 | TI         | @*258±5 | @*210±11 |
Appendix 24:

<table>
<thead>
<tr>
<th>Group</th>
<th>Hyp</th>
<th>H/R</th>
<th>Group</th>
<th>Hyp</th>
<th>H/R</th>
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</thead>
<tbody>
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<td>#110±3</td>
<td>ET (0hr)</td>
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<td>*87±4</td>
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<td>*164±8</td>
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<td>DI</td>
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<td>DI</td>
<td>*143±6</td>
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<td>ET (1hr)</td>
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<td>*211±7</td>
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<td>ZI</td>
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<td>ZI</td>
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<td>*109±8</td>
<td>EI</td>
<td>*288±10</td>
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ROS production Following H/R Summary Data.
Legend: ROS: Reactive oxygen species; Con: Control; ET: Endothelin-1; Norm: Normoxia; 0hr: no reperfusion; 1hr: one hour reperfusion; 24hr: 24 hour reperfusion; No inh: No inhibitor; ZI: PKCλ inhibitor; DI: Rottlerin; EI: PKCε Inhibitor.
* p<0.05 vs. Con (0hr) No inh, # p<0.05 vs. ET (0hr) No inh.
Appendix 25:

<table>
<thead>
<tr>
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<th>PKC Activity (U/mg)</th>
<th>PKA Activity (U/mg)</th>
</tr>
</thead>
<tbody>
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<td>*4.3±0.3</td>
</tr>
<tr>
<td>ET</td>
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<td>*3.1±0.2</td>
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<tr>
<td>BOS</td>
<td>*5.3±0.5</td>
<td>*3.8±0.2</td>
</tr>
<tr>
<td>BOS+ET</td>
<td>*5.4±0.2</td>
<td>*3.9±0.3</td>
</tr>
</tbody>
</table>

PKC and PKA Activity Summary Data. Values expressed as arbitrary units per mg of protein.
Legend: PKC: Protein kinase C; PKA: Protein kinase A; Con: Control; ET: Endothelin-1; BOS: Bosentan.
PKC: * p<0.05 vs. Con 30 Norm, # p<0.05 vs. ET Norm.
Appendix 26:

<table>
<thead>
<tr>
<th></th>
<th>Ser-Akt/Total-Akt ratio</th>
<th>Thr-Akt/Total-Akt ratio</th>
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<td>Norm 0 hr 1 hr 24 hr</td>
<td>Norm 0 hr 1 hr 24 hr</td>
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</tr>
<tr>
<td>BOS</td>
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<td>^*0.97±0.06  ^*1.74±0.05</td>
</tr>
<tr>
<td>BOS+ET</td>
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<td>^*0.94±0.06  ^*1.77±0.05</td>
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<tr>
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<tr>
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Akt/PKB Phosphorylation Summary Data.
Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin.
* p<0.05 vs. Con Norm), # p<0.05 Con 0hr, ^ p<0.05 ET (0hr).
## eNOS Protein Expression

### (% Normoxia (%)

<table>
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<tr>
<th>Group</th>
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<th>1 hr</th>
<th>24 hr</th>
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<td>@#101±11</td>
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### eNOS Protein Expression Summary Data.

Legend: Con: Control; ET: Endothelin-1; Cal: Calphostin C; Chel: Chelerythrine; BOS: Bosentan; FOS: Forskolin.

* p<0.05 vs. Con Norm (24hr), # p<0.05 Con 0hr, @ p<0.05 ET (0hr).
Appendix 28:

<table>
<thead>
<tr>
<th></th>
<th>Norm (30 min)</th>
<th>Norm (24hr)</th>
<th>0 hr</th>
<th>1 hr</th>
<th>24 hr</th>
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Caveolin-1 Expression Summary Data.
Legend: Con: Control; ET: Endothelin-1; BOS: Bosentan; Cal: Calphostin C; Chel: Chelerythrine; FOS: Forskolin
Appendix 29:

### PKCδ Translocation Summary Data

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<th>Group</th>
<th>Norm (30min)</th>
<th>Norm (24 hr)</th>
<th>0 hr</th>
<th>1 hr</th>
<th>24 hr</th>
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<tbody>
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PKCδ Translocation data.

Legend: Con: Control; ET: Endothelin-1; BOS: Bosentan; Cal: Calphostin C; Chel: Chelerythrine; FOS: Forskolin.

* p<0.05 Con Norm (24hr), # p<0.05 Con 0hr, @ p<0.05 ET 0hr

= p<0.05
Appendix 30:

<table>
<thead>
<tr>
<th>Group</th>
<th>Norm (30min)</th>
<th>Norm (24 hr)</th>
<th>0 hr</th>
<th>1 hr</th>
<th>24 hr</th>
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PKCε Translocation Summary Data.
Legend: Con: Control; ET: Endothelin-1; BOS: Bosentan; Cal: Calphostin C; Chel: Chelerythrine; FOS: Forskolin.
* p<0.05 Con Norm (24hr), # p<0.05 Con 0hr, @ p<0.05 ET 0hr
### Appendix 31:

**PKCλ Translocation (M/C Ratio)**

<table>
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<th>Norm (24 hr)</th>
<th>0 hr</th>
<th>1 hr</th>
<th>24 hr</th>
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<td>@*0.4±0.10</td>
<td>*0.3±0.04</td>
<td>*0.27±0.08</td>
<td>@*0.42±0.08</td>
<td>@#0.84±0.07</td>
</tr>
<tr>
<td>Chel</td>
<td>@#0.6±0.10</td>
<td>*0.4±0.06</td>
<td>*0.27±0.09</td>
<td>@*0.47±0.09</td>
<td>@#0.89±0.10</td>
</tr>
<tr>
<td>Chel+ET</td>
<td>@#0.5±0.20</td>
<td>*0.4±0.07</td>
<td>*0.26±0.10</td>
<td>@*0.45±0.10</td>
<td>@#0.87±0.11</td>
</tr>
<tr>
<td>PMA</td>
<td>@#2.1±0.20</td>
<td>@#2.0±0.20</td>
<td>@#0.49±0.13</td>
<td>@#0.71±0.13</td>
<td>@#0.89±0.10</td>
</tr>
<tr>
<td>PMA+ET</td>
<td>@#2.3±0.20</td>
<td>@#2.0±0.20</td>
<td>@#0.51±0.11</td>
<td>@#0.77±0.10</td>
<td>@#0.88±0.11</td>
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<tr>
<td>FOS</td>
<td>@#1.0±0.10</td>
<td>@#1.0±0.10</td>
<td>@#0.39±0.13</td>
<td>@#0.63±0.13</td>
<td>@#0.91±0.12</td>
</tr>
<tr>
<td>FOS+ET</td>
<td>@*0.4±0.10</td>
<td>*0.3±0.10</td>
<td>*0.25±0.10</td>
<td>@*0.54±0.12</td>
<td>@#0.84±0.09</td>
</tr>
<tr>
<td>H89</td>
<td>@#0.9±0.20</td>
<td>#0.9±0.10</td>
<td>@#0.38±0.11</td>
<td>@#0.66±0.09</td>
<td>@#0.93±0.11</td>
</tr>
<tr>
<td>H89+ET</td>
<td>@*0.4±0.10</td>
<td>*0.3±0.10</td>
<td>*0.28±0.12</td>
<td>@#0.55±0.11</td>
<td>@#0.81±0.13</td>
</tr>
</tbody>
</table>

**PKCλ Translocation Summary Data.**

Legend: Con: Control; ET: Endothelin-1; BOS: Bosentan; Cal: Calphostin C; Chel: Chelerythrine; FOS: Forskolin.

* p<0.05 Con Norm (24hr), # p<0.05 Con 0hr, @ p<0.05 ET 0hr
### eNOS Localization Summary Data

Legend: Con: Control; ET: Endothelin-1; BOS: Bosentan; Cal: Calphostin C; Chel: Chelerythrine; FOS: Forskolin.

* p<0.05 Con Norm (24hr), # p<0.05 Con 0hr, @ p<0.05 ET 0hr

<table>
<thead>
<tr>
<th>Group</th>
<th>Norm (30min)</th>
<th>Norm (24 hr)</th>
<th>0 hr</th>
<th>1 hr</th>
<th>24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>@#1.1±0.01</td>
<td>@#1.1±0.01</td>
<td>@*1.6±0.06</td>
<td>@#1.1±0.12</td>
<td>@#1.1±0.16</td>
</tr>
<tr>
<td>ET</td>
<td>@#1.7±0.05</td>
<td>@#1.9±0.05</td>
<td>@*1.9±0.07</td>
<td>@#1.2±0.09</td>
<td>@#1.2±0.07</td>
</tr>
<tr>
<td>BOS</td>
<td>@#1.2±0.1</td>
<td>@#1.1±0.1</td>
<td>@#1.0±0.13</td>
<td>@#1.1±0.07</td>
<td>@#1.0±0.11</td>
</tr>
<tr>
<td>BOS+ET</td>
<td>@#1.1±0.05</td>
<td>@#1.1±0.1</td>
<td>@#1.0±0.06</td>
<td>@#1.0±0.12</td>
<td>@#1.1±0.15</td>
</tr>
<tr>
<td>SH5</td>
<td>@#1.3±0.1</td>
<td>@#1.4±0.1</td>
<td>@*1.7±0.05</td>
<td>@#1.1±0.10</td>
<td>@#1.1±0.14</td>
</tr>
<tr>
<td>SH5+ET</td>
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<td>@#2.5±0.2</td>
<td>@*1.9±0.10</td>
<td>@#1.1±0.09</td>
<td>@#1.2±0.10</td>
</tr>
<tr>
<td>Cal</td>
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<td>@#1.5±0.09</td>
<td>@*1.7±0.07</td>
<td>@#1.1±0.08</td>
<td>@#1.2±0.12</td>
</tr>
<tr>
<td>Cal+ET</td>
<td>@#2.0±0.08</td>
<td>@#2.0±0.08</td>
<td>@*1.9±0.10</td>
<td>@#1.2±0.18</td>
<td>@#1.1±0.11</td>
</tr>
<tr>
<td>Chel</td>
<td>@#1.4±0.1</td>
<td>@#1.4±0.2</td>
<td>@*1.6±0.08</td>
<td>@#1.1±0.14</td>
<td>@#1.1±0.13</td>
</tr>
<tr>
<td>Chel+ET</td>
<td>@#2.0±0.07</td>
<td>@#2.1±0.08</td>
<td>@*1.9±0.10</td>
<td>@#1.2±0.11</td>
<td>@#1.1±0.09</td>
</tr>
<tr>
<td>PMA</td>
<td>@#0.8±0.06</td>
<td>@#0.9±0.05</td>
<td>@#1.5±0.14</td>
<td>@#1.1±0.11</td>
<td>@#1.1±0.08</td>
</tr>
<tr>
<td>PMA+ET</td>
<td>@#1.0±0.08</td>
<td>@#1.2±0.1</td>
<td>@#1.8±0.10</td>
<td>@#1.0±0.08</td>
<td>@#1.0±0.09</td>
</tr>
<tr>
<td>FOS</td>
<td>@#1.1±0.1</td>
<td>@#1.1±0.2</td>
<td>@#1.6±0.11</td>
<td>@#1.1±0.17</td>
<td>@#1.1±0.08</td>
</tr>
<tr>
<td>FOS+ET</td>
<td>@#1.7±0.1</td>
<td>@#1.8±0.1</td>
<td>@*1.9±0.13</td>
<td>@#1.1±0.16</td>
<td>@#1.1±0.08</td>
</tr>
<tr>
<td>H89</td>
<td>@#1.1±0.1</td>
<td>@#1.1±0.1</td>
<td>@#1.5±0.11</td>
<td>@#1.1±0.19</td>
<td>@#1.1±0.10</td>
</tr>
<tr>
<td>H89+ET</td>
<td>@#1.8±0.1</td>
<td>@#1.9±0.1</td>
<td>@#1.8±0.07</td>
<td>@#1.1±0.08</td>
<td>@#1.0±0.06</td>
</tr>
</tbody>
</table>

= p<0.05
Appendix 33:

<table>
<thead>
<tr>
<th>Group</th>
<th>8-Isoprostane (%Control)</th>
<th>ROS (%Baseline)</th>
<th>ET-1 Levels (fmole/L)</th>
<th>CyA Levels (ng/ml)</th>
<th>SRL Levels (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>#2 ±5%</td>
<td>#100%</td>
<td>1.0 ± 0.1</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>CyA</td>
<td>&amp;*50 ±2%</td>
<td>*172 ±8%</td>
<td>0.9 ± 0.1</td>
<td>60 ± 11</td>
<td>--</td>
</tr>
<tr>
<td>SRL</td>
<td>#3 ±3%</td>
<td>#114 ±6%</td>
<td>#*0.4 ± 0.1</td>
<td>--</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>BH₄+CyA</td>
<td>#*-10 ±1%</td>
<td>--</td>
<td>#*0.5 ± 0.1</td>
<td>61 ± 8</td>
<td>--</td>
</tr>
<tr>
<td>BH₄</td>
<td>&amp;#*-28 ±3%</td>
<td>--</td>
<td>#*0.6 ± 0.1</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>BOS+CyA</td>
<td>#*-4 ±11%</td>
<td>#106 ±6%</td>
<td>#*0.8 ± 0.03</td>
<td>58 ± 10</td>
<td>--</td>
</tr>
<tr>
<td>BOS</td>
<td>#0.3 ±2%</td>
<td>&amp;#*76 ±5%</td>
<td>#*0.6 ± 0.1</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>BOS+BH₄+CyA</td>
<td>#*-5 ±6%</td>
<td>--</td>
<td>#*0.7 ± 0.1</td>
<td>55 ± 9</td>
<td>--</td>
</tr>
<tr>
<td>BOS+BH₄</td>
<td>#*-12 ±3%</td>
<td>--</td>
<td>#*0.6 ± 0.02</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Reactive Oxygen Species and Plasma Level Summary Data.
* p<0.05 vs Con, # p<0.05 CyA, & p<0.05 vs. all other groups,
Appendix 34:

<table>
<thead>
<tr>
<th>Group</th>
<th>Ach</th>
<th></th>
<th>SNP</th>
<th></th>
<th>ET</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Emax%</td>
<td>ED₅₀ (x10⁻⁷M/L)</td>
<td>Emax%</td>
<td>ED₅₀ (x10⁻⁷M/L)</td>
<td>%Cmax</td>
</tr>
<tr>
<td>Con</td>
<td>^#52%±8%</td>
<td>#2.0±0.5</td>
<td>61%±7%</td>
<td>^#3.2±1.0</td>
<td>#211%±9%</td>
</tr>
<tr>
<td>CyA</td>
<td>^*24%±7%</td>
<td>#*3.2±0.3</td>
<td>62%±10%</td>
<td>^#6.3±1.2</td>
<td>*260%±10%</td>
</tr>
<tr>
<td>SRL</td>
<td>^#58%±4%</td>
<td>&amp;#*0.8±0.2</td>
<td>65%±5%</td>
<td>^#2.5±1.1</td>
<td>#217%±8%</td>
</tr>
<tr>
<td>BH₄+CyA</td>
<td>^#50%±8%</td>
<td>#*2.5±0.4</td>
<td>67%±11%</td>
<td>^#4.2±0.3</td>
<td>*268%±5%</td>
</tr>
<tr>
<td>BH₄</td>
<td>^#54%±8%</td>
<td>#2.0±0.1</td>
<td>#*72%±3%</td>
<td>^#2.0±0.4</td>
<td>*241%±8%</td>
</tr>
<tr>
<td>BOS+CyA</td>
<td>^#53%±4%</td>
<td>#*1.3±0.3</td>
<td>66%±7%</td>
<td>^#2.8±0.5</td>
<td>@#*158%±28%</td>
</tr>
<tr>
<td>BOS</td>
<td>^#54%±4%</td>
<td>#*1.3±0.3</td>
<td>62%±8%</td>
<td>^#1.4±0.6</td>
<td>@#*146%±5%</td>
</tr>
<tr>
<td>BOS+BH₄+CyA</td>
<td>#*61%±5%</td>
<td>#*1.0±0.5</td>
<td>#*72%±7%</td>
<td>^#0.9±0.7</td>
<td>@#*155%±15%</td>
</tr>
<tr>
<td>BOS+BH₄</td>
<td>#*63%±3%</td>
<td>#*1.5±0.3</td>
<td>#*76%±4%</td>
<td>^#1.0±0.8</td>
<td>@#*163%±5%</td>
</tr>
</tbody>
</table>

Vasomotor Function Summary Data.
* p<0.05 vs Con, # p<0.05 CyA, & p<0.05 vs. all other groups, @ p<0.05 vs. all non-BOS treated groups,
^ p<0.05 vs. BOS+BH₄, BOS+BH₄+CyA
Appendix 35:

<table>
<thead>
<tr>
<th>Group</th>
<th>( \text{ET}_A \text{Rc} )</th>
<th>( \text{ET}_B \text{Rc} )</th>
<th>eNOS</th>
<th>TNF( \alpha )</th>
<th>TGF( \beta )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>#100%</td>
<td>100%</td>
<td>#100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>CyA</td>
<td>&amp;*133 ± 5%</td>
<td>112 ± 13%</td>
<td>*72 ± 4%</td>
<td>117 ± 12%</td>
<td>104 ± 17%</td>
</tr>
<tr>
<td>SRL</td>
<td>#102 ± 3%</td>
<td>102 ± 4%</td>
<td>#99 ± 6%</td>
<td>102 ± 13%</td>
<td>100 ± 18%</td>
</tr>
<tr>
<td>BH(_4)+CyA</td>
<td>#99 ± 3%</td>
<td>#*122 ± 6%</td>
<td>#*90 ± 3%</td>
<td>106 ± 13%</td>
<td>104 ± 14%</td>
</tr>
<tr>
<td>BH(_4)</td>
<td>#98 ± 4%</td>
<td>#*134 ± 4%</td>
<td>#102 ± 5%</td>
<td>112 ± 17%</td>
<td>103 ± 16%</td>
</tr>
<tr>
<td>BOS+CyA</td>
<td>#106 ± 2%</td>
<td>103 ± 6%</td>
<td>#*88 ± 6%</td>
<td>105 ± 11%</td>
<td>101 ± 13%</td>
</tr>
<tr>
<td>BOS</td>
<td>#103 ± 6%</td>
<td>105 ± 4%</td>
<td>&amp;#*193 ± 11%</td>
<td>109 ± 9%</td>
<td>99 ± 14%</td>
</tr>
<tr>
<td>BOS+BH(_4)+CyA</td>
<td>#103 ± 3%</td>
<td>102 ± 7%</td>
<td>#93 ± 3%</td>
<td>108 ± 14%</td>
<td>105 ± 12%</td>
</tr>
<tr>
<td>BOS+BH(_4)</td>
<td>#106 ± 6%</td>
<td>107 ± 5%</td>
<td>#*116 ± 6%</td>
<td>106 ± 10%</td>
<td>102 ± 15%</td>
</tr>
</tbody>
</table>

Quantitative Western Blot Analysis Summary Data.
* p<0.05 vs Con, # p<0.05 CyA, & p<0.05 vs. all other groups,