VASCULOPROTECTIVE EFFECTS OF INSULIN AND RESVERATROL IN VIVO

By

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A thesis submitted in conformity with the requirements
For the degree of Doctor of Philosophy

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General Abstract

Atherosclerosis is a leading cause of morbidity and mortality worldwide and type 2 diabetes and obesity-associated metabolic syndrome, both characterized by insulin resistance, are potent risk factors. These conditions also increase the risk for restenosis after revascularization procedures used for treatment of atherosclerosis. Studies have shown that insulin and resveratrol (RSV), a red wine polyphenol, decrease neointimal growth after vessel injury in models of restenosis, demonstrating a protective effect on the vasculature. However, oral glucose and sucrose were used in insulin studies to maintain normoglycemia, and their effect on neointimal formation was not assessed. Several studies have shown that nitric oxide (NO) production is stimulated by insulin and RSV, and since NO can decrease neointimal growth, the objective of this thesis was to address the mechanism of action of insulin or RSV to protect against restenosis, and determine whether NO production mediates these effects. To examine this, we treated rats with insulin or RSV and performed arterial balloon injury.

In Study 1, insulin reduced neointimal area after injury in rats receiving oral glucose but not oral sucrose. Oral glucose alone had no effect on neointimal formation or insulin sensitivity whereas oral sucrose increased neointimal growth and induced insulin resistance. In Study 2, insulin decreased neointimal area and cell migration, and increased re-endothelialization. These effects were abolished by nitric oxide synthase (NOS) inhibition. In addition, insulin increased eNOS protein expression in the vessel. In Study 3, RSV reduced neointimal growth, cell proliferation, and migration after injury, without affecting re-endothelialization. Most of these effects were abolished by NOS inhibition, except for the decrease in cell migration. Insulin sensitivity and systolic blood pressure were not affected by RSV.
Together, the results demonstrate that insulin, independent of glycemic effects, and RSV have a protective effect on the vessel against restenosis, which is mediated by NO. Since both insulin and RSV decrease neointimal formation without negatively impacting re-endothelialization, insulin or RSV treatment could provide some advantage over anti-mitogenic agents currently used in drug-eluting stents, which delay re-endothelialization. These studies suggest that insulin or RSV may have clinical potential in the prevention of restenosis after angioplasty.
Acknowledgment

This thesis is the result of five years of work whereby I have been supported by numerous people and I am pleased to now have the opportunity to express my appreciation for all of them. First and foremost I offer my sincerest gratitude to my supervisor, Dr Adria Giacca, who has supported me throughout my doctoral studies with her motivation and knowledge, allowing me the room to work in my own way. I simply could not wish for a friendlier or more generous supervisor, and your dedication to research and teaching is an inspiration.

I would like to thank my co-supervisor Dr Evangelia Tsiani, and committee members Dr Michelle Bendeck, and Dr George Fantus who monitored my work and took tremendous effort in reading and providing me with valuable comments on several versions of this thesis. I thank you all.

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# Table of Contents

1.0 Introduction ........................................................................................................ 1

1.1 Atherosclerosis ........................................................................................................ 1

1.2 Mechanism of Atherosclerosis Development ............................................................ 2

1.3 Restenosis ................................................................................................................. 3

1.4 Animal Models of Restenosis .................................................................................... 4

1.5 Mechanism of the Arterial Response to Balloon Catheter Injury .............................. 6

1.5.1 Platelet Activation ........................................................................................................ 6

1.5.2 Leukocyte Infiltration ................................................................................................ 6

1.5.3 Vascular Smooth Muscle Cell Phenotype Switch .................................................... 7

1.5.4 Vascular Smooth Muscle Cell Proliferation and Apoptosis ........................................ 8

1.5.5 Vascular Smooth Muscle Cell Migration ........................................................................ 9

1.5.6 Matrix Metalloproteinases (MMPs) ............................................................................ 12

1.5.7 Extracellular Matrix .................................................................................................. 13

1.5.8 Endothelial Regeneration .......................................................................................... 14

1.5.9 Progenitor Cell Contribution ...................................................................................... 15

1.6 Type 2 Diabetes and Metabolic Syndrome ............................................................... 18

1.7 Nitric Oxide .............................................................................................................. 20

1.8 Insulin ....................................................................................................................... 24

1.8.1 General effects of insulin .......................................................................................... 24

1.8.2 Effects of insulin on atherosclerosis .......................................................................... 25

1.8.3 Effects of insulin on restenosis .................................................................................. 27

1.9 Mechanism of Insulin Action in the Vasculature ....................................................... 30

1.9.1 General features of insulin signal transduction pathways ........................................ 30

1.9.2 Insulin stimulates NO production and induces vasodilation ..................................... 31

1.9.3 Insulin stimulates ET-1 and induces vasoconstriction .............................................. 32

1.9.4 Insulin inhibits platelet function .............................................................................. 33

1.9.5 Insulin has anti-inflammatory effects .................................................................... 33

1.9.6 Effect(s) of insulin on proliferation and migration ..................................................... 34

1.10 Resveratrol ............................................................................................................ 35

1.10.1 General effects of RSV .......................................................................................... 35

1.10.2 Effects of resveratrol on atherosclerosis .................................................................. 36

1.10.3 Effects of resveratrol on restenosis ......................................................................... 37

1.11 Mechanism of Resveratrol Action in the Vasculature ............................................. 37

1.11.1 General resveratrol signal transduction .................................................................. 37

1.11.2 Resveratrol stimulates NO production and induces vasodilation ............................ 38

1.11.3 Resveratrol inhibits platelet function and thrombus formation .................................. 39

1.11.4 Resveratrol has anti-oxidant effects ...................................................................... 40

1.11.5 Resveratrol has anti-inflammatory effects ................................................................. 40

1.11.6 Resveratrol inhibits proliferation and migration ....................................................... 41

1.12 Summary ............................................................................................................... 43

1.13 Rationale and Significance of the Studies .............................................................. 43

1.14 General Hypothesis ............................................................................................... 45

1.15 Specific Aims .......................................................................................................... 45

2.0 General Methods ...................................................................................................... 48

2.1 Animals .................................................................................................................... 48

2.2 Surgical Procedures ............................................................................................... 48

2.2.1 Subcutaneous Implant Procedure ............................................................................ 48
2.2.2 Intraperitoneal Catheterization for Glucose Infusion .......................................................... 49
2.2.3 Osmotic Pump Implant Procedure .................................................................................. 49
2.2.4 Carotid Balloon Injury ................................................................................................. 50
2.2.5 Aortic Balloon Injury ................................................................................................. 50
2.2.6 Vessel Cannulation .................................................................................................... 50

2.3 Blood and Vessel Sample Collection ............................................................................... 51
   2.3.1 Fixed Vessel Collection .......................................................................................... 51
   2.3.2 Frozen Vessel Collection ....................................................................................... 52

2.4 Morphometric Measurements and Calculations .............................................................. 52

2.5 Histomorphometry/Matrix Staining .................................................................................. 53
   2.5.1 Cell Migration Assay .............................................................................................. 53
   2.5.2 BrdU- Labelling ....................................................................................................... 54
   2.5.3 Terminal dUTP nick- end labeling (TUNEL) Assay ...................................................... 55
   2.5.4 Extracellular Matrix Staining with Movat’s Pentachrome and Picrosirius Red ......... 56
   2.5.5 Evan’s Blue Staining ................................................................................................ 57

2.6 Vessel Homogenization .................................................................................................... 58

2.7 Protein Assay .................................................................................................................. 58

2.8 Western Blot Analysis ..................................................................................................... 59

2.9 Flow Cytometry .............................................................................................................. 60

2.10 Hyperinsulinemic- Euglycemic Clamp ............................................................................ 60

2.11 Blood Pressure Analysis ................................................................................................ 61

2.12 Metabolic Parameters .................................................................................................... 61
   2.12.1 Plasma Insulin ........................................................................................................ 61
   2.12.2 Plasma Free Fatty Acid ......................................................................................... 62
   2.12.3 Plasma Triglyceride ............................................................................................... 63
   2.12.4 Plasma Glucose Determination ............................................................................ 64

2.13 Statistical Analysis ........................................................................................................ 65

3.0 Study 1 ............................................................................................................................. 66
   3.1 Abstract ......................................................................................................................... 67
   3.2 Introduction .................................................................................................................. 68

3.3 Materials and Methods ................................................................................................... 70
   3.3.1 Animal Models ........................................................................................................ 70
   3.3.2 Surgical Procedures ............................................................................................... 70
   3.3.3 Vessel and Blood Sample Collection ...................................................................... 71
   3.3.4 Metabolic Parameters ............................................................................................ 71
   3.3.5 Histomorphometry/Matrix Staining ....................................................................... 71
   3.3.6 Vessel Cannulation ............................................................................................... 72
   3.3.7 Hyperinsulinemic- Euglycemic Clamp ..................................................................... 72
   3.3.8 Statistical Analysis .................................................................................................. 72

3.4 Results .................................................................................................................................. 72
   3.4.1 Metabolic Parameters ............................................................................................. 72
   3.4.2 Morphometric Measurements ............................................................................... 74
   3.4.3 Insulin Sensitivity Measurements ........................................................................... 75

3.5 Discussion ......................................................................................................................... 75
   3.5.1 Effect of insulin and oral glucose ............................................................................ 76
   3.5.2 Effect of insulin and oral sucrose ............................................................................ 77

4.0 Study 2 .............................................................................................................................. 89
   4.1 Abstract ......................................................................................................................... 90
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AhR</td>
<td>aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>AICAR</td>
<td>5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside</td>
</tr>
<tr>
<td>AMPK</td>
<td>5′ adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ApoE</td>
<td>apolipoprotein E</td>
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<td>AP-1</td>
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<td>cyclin dependent kinase</td>
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<td>cyclic guanylate monophosphate</td>
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<td>COX</td>
<td>cyclooxygenase</td>
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<td>cardiovascular disease</td>
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<td>DCCT</td>
<td>Diabetes Control and Complications Trial</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
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<td>epidermal growth factor</td>
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<td>endothelial nitric oxide synthase</td>
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<td>endothelial progenitor cell</td>
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<tr>
<td>ERK 1/2</td>
<td>extracellular regulated kinase</td>
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<td>fibroblast growth factor</td>
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<td>flavin mononucleotide</td>
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<td>forkhead transcription factor-1</td>
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<tr>
<td>JNK</td>
<td>c-jun NH2-terminal kinase</td>
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<td>LCCA</td>
<td>left common carotid artery</td>
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<td>LDL</td>
<td>low-density lipoprotein</td>
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<tr>
<td>LDLR</td>
<td>low-density lipoprotein receptor</td>
</tr>
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<td>L-NAME</td>
<td>L-NG-Nitroarginine methyl ester</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<td>MCP-1</td>
<td>monocyte chemoattractant protein-1</td>
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<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>MSC</td>
<td>mesenchymal stem cell</td>
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<td>MT-MMP</td>
<td>membrane type-matrix metalloproteinase</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate hydrogen</td>
</tr>
<tr>
<td>NFkB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>NMDA</td>
<td>N- methyl D- aspartate</td>
</tr>
<tr>
<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<td>polyethylene</td>
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<td>phosphatidylinositol 4, 5- bisphosphate</td>
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<td>phosphatidylinositol-3, 4, 5- triphosphate</td>
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<td>protein kinase C</td>
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<tr>
<td>PKG</td>
<td>protein kinase G</td>
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<td>PSR</td>
<td>picrosirius red</td>
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<td>PTCA</td>
<td>percutaneous transluminal coronary angioplasty</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
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<tr>
<td>PTP1B</td>
<td>protein tyrosine phosphatase 1B</td>
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<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
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<td>RIA</td>
<td>radioimmunoassay</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<td>RSV</td>
<td>resveratrol</td>
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<td>SDS</td>
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<td>SHIP-2</td>
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<td>smooth muscle-myosin heavy chain</td>
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<td>smooth muscle 22 alpha</td>
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<td>Sos</td>
<td>son of sevenless</td>
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<td>STZ</td>
<td>streptozotocin</td>
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<tr>
<td>S6K1</td>
<td>p70 S6 kinase-1</td>
</tr>
<tr>
<td>TBST</td>
<td>tris-buffered saline tween</td>
</tr>
<tr>
<td>TG</td>
<td>triglyceride</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal dUTP nick-end labeling</td>
</tr>
<tr>
<td>UKPDS</td>
<td>United Kingdom Prospective Diabetes Study</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low-density lipoprotein</td>
</tr>
<tr>
<td>VSMC</td>
<td>vascular smooth muscle cell</td>
</tr>
<tr>
<td>ZDF</td>
<td>Zucker diabetic fatty</td>
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</tbody>
</table>
List of Tables

Table 3.1. Neointimal formation with or without an i.p. catheter.................................79

Table 3.2. Daily fasting and fed plasma glucose levels over the treatment period..............80

Table 3.3. Fasting plasma levels of insulin, triglycerides, and free fatty acids at 14 days after arterial injury..............................................................81

Table 3.4. Fed plasma levels of insulin, triglycerides, and free fatty acids at 14 days after arterial injury...............................................................................82

Table 3.5. Intimal cell number and cell density per unit area measured at 14 days after vessel injury.....................................................................................83

Table 4.1. Daily fasting and fed plasma glucose levels over the treatment period.............101

Table 4.2. Fed plasma levels of insulin, triglycerides, and free fatty acids at 28 days after arterial injury..............................................................102

Table 4.3. Percentage of white blood cells stained positive for c-kit, sca-1, and VEGFR2.....103

Table 5.1. Daily food, fluid and total caloric intake, final weight gain, fed plasma glucose and insulin levels over the treatment periods...............................123
List of Figures

Figure 1.1. Signal transduction by the insulin receptor. .............................................................. 46

Figure 1.2. Proposed signal transduction by resveratrol.............................................................. 47

Figure 3.1. The effect of insulin, oral glucose, and oral sucrose on neointimal formation. ...... 84

Figure 3.2. The effect of insulin, oral glucose, and oral sucrose on intimal area. ......................... 85

Figure 3.3. The effect of insulin, oral glucose, and oral sucrose on medial area and external elastic lamina perimeter. ............................................................................................................... 86

Figure 3.4. The effect of insulin, oral glucose, and oral sucrose on cell proliferation. .............. 87

Figure 3.5. The effect of oral glucose and oral sucrose on whole body insulin sensitivity. ....... 88

Figure 4.1. The effect of insulin and NOS inhibition on intimal area. ..................................... 104

Figure 4.2. The effect of insulin and NOS inhibition on elastin and collagen accumulation. ... 105

Figure 4.3. The effect of insulin and NOS inhibition on cell migration. ................................. 106

Figure 4.4. The effect of insulin on SMC differentiation markers and eNOS expression........ 107

Figure 4.5. The effect of insulin and NOS inhibition on re-endothelialization. ...................... 108

Figure 4.6. The effect of insulin and NOS inhibition on systolic blood pressure...................... 109

Figure 5.1. The effect of RSV and NOS inhibition on intimal area. ........................................ 124

Figure 5.2. The effect of RSV and NOS inhibition on cell proliferation................................. 125

Figure 5.3. The effect of RSV on cell apoptosis....................................................................... 126

Figure 5.4. The effect of RSV and NOS inhibition on elastin and collagen content................. 127

Figure 5.5. The effect of RSV and NOS inhibition on cell migration. .................................... 128

Figure 5.6. The effect of RSV on re-endothelialization.......................................................... 129

Figure 5.7. The effect of RSV and NOS inhibition on systolic blood pressure.......................... 130

Figure 5.8. The effect of RSV on whole body insulin sensitivity............................................. 131

Figure 7.1. Summary of the effects of insulin and RSV in the vessel after arterial balloon injury. ..................................................................................................................................................... 143
Published Manuscripts Arising from Completion of this Thesis

**Introduction Chapter** Breen DM, Giacca A. *Effects of Insulin on the Vasculature.* (Current Vascular Pharmacology, In Revision #183846).

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**Note:** For Study 2, Table 4.3 and Figure 4.4A-F have been published in the manuscript listed above. The remaining data presented in Study 2 are currently unpublished.


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Introduction

1.1 Atherosclerosis

Atherosclerotic cardiovascular disease (CVD) (coronary heart disease, myocardial infarction, stroke, peripheral vascular disease) is the leading cause of morbidity and mortality in the developed world, accounting for approximately 35.3% of all deaths in 2005. The Framingham Heart Study, which analyzed the 10 year incidence of atherosclerotic CVD and death in men, found a decline in both morbidity and mortality due to cardiovascular diseases. This may be explained by improved primary prevention and medical interventions. However, due to the recent epidemic of obesity and obesity-associated diabetes, diabetes-associated cardiovascular mortality is increasing and is therefore a major and growing public health concern. Atherosclerosis develops over a lifetime, and often symptoms or complications are not present. However, when clinical complications occur, they are highly variable and range from chronic stable symptoms, such as coronary heart disease, angina or arteriosclerosis, to fatal acute events, such as myocardial infarction or stroke. Non-modifiable risk factors for atherosclerosis include age, gender, and family history of CVD. Modifiable risk factors include smoking, alcohol consumption, hypertension, hyperlipidemia, diabetes, insulin resistance, and obesity. Methods for prevention have included identification of risk factors for an atherosclerotic event, lifestyle modifications, and pharmacologic intervention to lower plasma cholesterol concentrations.
1.2 Mechanism of Atherosclerosis Development

Atherosclerosis represents a chronic inflammatory response of the arterial wall\textsuperscript{4}. The atherosclerotic lesion is divided into three stages: 1) the fatty streak; 2) the intermediate lesion; and 3) the fibrous plaque. The fatty streak is formed by the accumulation of macrophages and T-lymphocytes within the arterial wall, which may begin in childhood. The intermediate lesion is characterized by the accumulation of lipids, vascular smooth muscle cells (VSMC), and extracellular matrix (ECM). The lesion progressively increases in size and as a result the lumen of the vessel narrows. Over time an advanced fibrous plaque develops, which is characterized by a lipid-rich core containing necrotic tissue covered by a fibrous cap. An unstable plaque eventually ruptures and leads to thrombosis, which is partial or complete occlusion of the blood vessel.

The pathogenesis of atherosclerosis is described by a more recent version of the ‘response to injury’ hypothesis that emphasizes endothelial dysfunction and is widely accepted\textsuperscript{4}. Endothelial dysfunction is defined as the loss of endothelial integrity and/ or function. The normal healthy endothelium inhibits vasoconstriction, provides an anti-thrombotic surface to prevent platelet and leukocyte adhesion, and inhibits VSMC growth. Endothelial dysfunction is caused by mechanical or chemical injury; for example by oxidized low-density lipoproteins (LDL), chemical agents, toxins (including byproducts of cigarette smoking), hyperglycemia, and hyperhomocystinemia. The result is compensatory responses that change the normal homeostatic properties of the endothelium transforming it to a pro-thrombotic state, including impaired endothelial-dependent vasodilation.

The earliest stage of atherogenesis is characterized by proteoglycan-mediated lipoprotein retention in the subendothelium; where proteoglycans bind lipoproteins through ionic interaction and lipoproteins permeate the intact endothelium and become oxidized. This process initiates an inflammatory response where membrane adhesion molecule expression increases on endothelial cells, thereby promoting leukocyte and platelet adhesion and increased permeability to lipoproteins (reviewed in\textsuperscript{4}). Leukocytes migrate into the intima and become macrophages that engulf modified
forms of lipoproteins, especially LDL, thus becoming foam cells that form the fatty streak. The endothelium, macrophages, and platelets release cytokines and growth factors, which stimulate migration and proliferation of VSMCs that mix with the area of inflammation forming an intermediate lesion. Progression of inflammation results in focal necrosis. VSMC proliferation and matrix deposition results in fibrosis. At this point the lesion is composed of a lipid and necrotic tissue core covered by a fibrous cap and the lesion protrudes into the lumen and alters blood flow (reviewed in\textsuperscript{4}). Collagen provides strength and stability to the fibrous cap, however thin, macrophage- rich caps are unstable and can promote plaque rupture. The majority of sudden deaths from myocardial infarcts are attributed to ruptures or fissures resulting in hemorrhage into the plaque, thrombosis, and occlusion of the artery\textsuperscript{4}.

1.3 Restenosis

In contrast to atherosclerosis, restenosis is initiated by acute mechanical injury, such as balloon angioplasty, and the development is rapid\textsuperscript{5}. Percutaneo us transluminal coronary angioplasty (PTCA) is a well- established technique for myocardial revascularization of patients with coronary artery disease due to atherosclerosis. However, PTCA alone is limited by restenosis in 30- 60% of cases\textsuperscript{6}. Therefore, stenting after PTCA was introduced using the balloon- expandable bare- metal stent as it provided distinct advantages over angioplasty alone, such as a drastically reduced rate of restenosis (20- 30% in humans)\textsuperscript{6}. Subsequently, drug- eluting stents were designed to release pharmacological agents after insertion to inhibit VSMC proliferation and in turn restenosis. First- generation drug- eluting stents were coated with either rapamycin or paclitaxel, which reduced the restenosis rate to less than 10% as compared to the bare- metal stents\textsuperscript{7}. These agents primarily inhibit VSMC migration and proliferation, which represent crucial events in the development of in- stent restenosis\textsuperscript{8}. Stent thrombosis has emerged as a severe complication\textsuperscript{9}; despite this, PTCA with stenting is currently the preferred method of
treatment. Although drug-eluting stents have greatly decreased the rate of restenosis, results in diabetic individuals are still worse than results in non-diabetic people\textsuperscript{10}.

Restenosis is the renarrowing of the blood vessel after balloon angioplasty and is a function of early elastic recoil, negative vessel remodeling, and neointimal hyperplasia\textsuperscript{11}. Elastic recoil is the rapid loss of lumen gain after balloon angioplasty due to the elastin fibers that comprise the internal and external elastic lamina. Vessel remodelling is a gradual process by which lumen size is structurally modified in response to changes in blood flow\textsuperscript{11}. For example, in initial stages of atherosclerosis the vessel enlarges (positive remodeling) to increase lumen size in response to the growing intima. However, severe atherosclerosis in vessels promotes negative or constrictive remodeling, which further decreases lumen size. Neointimal hyperplasia is an exaggerated healing response that occurs in the vessel wall after injury. In humans, the neointima after balloon injury stabilizes in three to six months\textsuperscript{12}. Elastic recoil and constrictive remodeling can be eliminated with a stent, therefore restenosis after stenting is mainly characterized by neointimal formation, which is a complex process resulting from VSMC proliferation and deposition of ECM\textsuperscript{13}.

1.4 Animal Models of Restenosis

Animal models are important tools in understanding the vascular response to arterial injury following interventions, especially balloon angioplasty. The most commonly used animal models for the study of restenosis after balloon angioplasty are the primate, pig, rabbit, rat, and mouse. Primate models best resemble human restenosis and thrombotic activity since they are prone to acute stent thrombosis within the first three days following injury\textsuperscript{14}. In the porcine model both balloon angioplasty and stenting result in extensive neointimal formation. In addition, this model is useful to study the kinetics of restenosis as they develop human-like lesions\textsuperscript{14}. The rabbit model has been used to study dyslipidemia and restenosis as hypercholesterolemia can be readily induced using atherogenic diets, resulting in reproducible
atherosclerotic-like lesions\textsuperscript{15}. Transgenic mouse models are also used to study the molecular mechanisms involved in neointimal growth, however balloon catheters are too large to be used in mice, so arterial injury is induced with small guidewires\textsuperscript{16}. The kinetics of neointimal formation are similar to the rat model, however neointimal formation in mice is often difficult to reproduce due to the nature of the injury with the irregular shape of the guidewire and also the different mouse strains\textsuperscript{16}.

Although the rat model has minimal inflammatory and thrombotic components, and does not allow the study of long-term vessel remodelling, it is the best characterized model of arterial injury and restenosis (reviewed in \textsuperscript{17}). In this thesis both aortic and left common carotid arterial angioplasty models were used, which involve inducing fibroproliferative lesions within a long segment of the artery using a 2 French Fogarty catheter. Balloon angioplasty induces intimal injury by denuding the endothelial layer, resulting in platelet activation and secretion of growth factors from platelets and damaged VSMCs. Inflammation can also occur due to monocyte recruitment to the site of injury. Subsequently, there is VSMC migration from media to intima, and VSMC proliferation and ECM deposition in the neointima, resulting in lumen narrowing. Bone marrow derived smooth muscle precursors can also contribute to neointimal growth\textsuperscript{18}, as can adventitial cells\textsuperscript{19}. Re-endothelialization occurs starting from endothelium at the margins of the denuded area and is contributed by bone marrow derived endothelial precursor cells\textsuperscript{20}. The time course of neointimal growth has been well established after experimental balloon angioplasty in the rat (reviewed in \textsuperscript{17}). Medial VSMC proliferation peaks two days after balloon angioplasty and is followed by a peak in VSMC migration to the intima at four days. VSMCs replicate in the neointima and reach maximum proliferation by seven days. Neointimal growth, mainly due to ECM deposition, continues until 28 days at which time lesion growth plateaus. The rat arterial injury model is limited when compared with human restenosis because; 1) inflammation and thrombosis are implicated in human restenosis and are much less after vessel
injury in the rat\textsuperscript{21}, 2) in humans there is compensatory enlargement of the vessel to maintain constant lumen diameter, however this does not occur in rats\textsuperscript{17}, and 3) there are no pre-existing lesions in the rat model of balloon injury as in humans at the time of PTCA. As compounds that reduce restenosis in the rat model are not always effective in humans, particularly anti-oxidant and anti-inflammatory treatments, interpretation of results obtained in the rat model of balloon injury should be viewed with caution.

1.5 Mechanism of the Arterial Response to Balloon Catheter Injury

1.5.1 Platelet Activation

The role of platelets in promoting neointimal formation in the rat model of balloon injury has been demonstrated\textsuperscript{22}. Endothelial denudation of the vessel exposes the subendothelial components, mainly collagen and fibronectin, to the circulating blood, thereby promoting platelet adhesion and aggregation within seconds after endothelial loss and up to seven days after injury\textsuperscript{22}. Binding of platelets to the components of subendothelium matrix proteins, results in degranulation of the platelets and release of prothrombotic factors such as thrombin, ADP, serotonin, thromboxane A\textsubscript{2}, fibrinogen, and von Willebrand factor, which promote additional adhesion and aggregation\textsuperscript{11}. Platelet- derived growth factor (PDGF) is also released at the site of injury and may stimulate VSMC activation\textsuperscript{23}.

1.5.2 Leukocyte Infiltration

Leukocytes and monocytes are also recruited to the vessel wall following balloon injury and have been implicated in neointimal formation\textsuperscript{24}. Leukocytes secrete many cytokines and growth factors that promote neointimal growth\textsuperscript{25}. Monocyte- derived macrophages, which secrete growth factors and cytokines that promote VSMC proliferation\textsuperscript{26}, comprise approximately 1\% of the total neointimal cell number\textsuperscript{21}. Taken together, these studies suggest a role of inflammation in the vessel response to balloon injury leading to neointimal formation.
1.5.3 Vascular Smooth Muscle Cell Phenotype Switch

Immediately following arterial injury, the majority of medial VSMCs (up to 70%) undergo apoptosis, which will be further discussed in the following section. In the 48 hours following arterial injury, VSMC dedifferentiation is promoted by exposure to growth factors such as PDGF, fibroblast growth factor (FGF), epidermal growth factor (EGF), and TGFβ. In contrast, studies suggest that insulin-like growth factor-1 (IGF-1) may promote VSMC differentiation as it also strongly activates phosphatidylinositol-3 kinase (PI3K)/Akt signalling in addition to mitogen-activated protein kinase (MAPK), which is the main mechanism of action for the other growth factors. Normally quiescent and differentiated VSMCs become active, enter the cell cycle, and divide (between 10-30% of surviving VSMCs). The phenotype switch to an activated secretory state results in increased synthesis of cytokines and growth factors that act as paracrine mediators to activate additional VSMCs, facilitate leukocyte chemotaxis and infiltration into the vessel wall, and stimulate production of collagen and elastin. Activated VSMCs also proliferate and migrate to the intima where the inflammatory components further promote the activated state. Activated VSMCs are characterized by changes in morphology with decreased cytoskeletal myofilaments, reduced levels of contractile proteins, and increased synthetic organelles. Smooth muscle myosin heavy chain (SM-MHC), smooth muscle 22 alpha (SM22α), and SMC α-actin are protein markers of differentiation and their protein expression decreases as VSMCs dedifferentiate. VSMC activation and phenotypic switch is reversible.

Activation of the mammalian target of rapamycin (mTOR)/p70S6 kinase-1 (S6K1) pathway, which is well known to promote proliferation and protein synthesis, stimulates VSMC dedifferentiation by decreasing contractile protein expression (SM-MHC, calponin, and SMC α-actin), resulting in a more active proliferative state.
1.5.4 Vascular Smooth Muscle Cell Proliferation and Apoptosis

As mentioned in the previous section, during the initial 48 hours following arterial injury, stationary and quiescent VSMCs enter the cell cycle and divide, resulting in restoration of the number of VSMCs in the medial layer where eventually they resume their quiescent state. Medial VSMC proliferation begins as early as 24 hours after arterial injury and peaks at two days, followed by VSMC migration to the intima (which will be discussed in the next section) where VSMCs replicate and reach maximum proliferation by seven days (reviewed in\textsuperscript{17}). VSMCs proliferate under the influence of various mediators including oxidative stress\textsuperscript{35}, growth factors released from leukocytes and platelets, and interactions of VSMCs with the ECM\textsuperscript{36}. Initial medial VSMC proliferation is mediated by FGF in this rat arterial injury model\textsuperscript{37}. FGF is found in VSMCs and endothelial cells\textsuperscript{37} and also targets fibroblasts and endothelial cells to stimulate proliferation in contrast to vascular endothelial growth factor (VEGF), which stimulates proliferation primarily in endothelial cells. This will be described in more detail in section 1.5.8. In addition, expression of IGF-1\textsuperscript{38} and its receptor\textsuperscript{39} are increased in the rat aorta after balloon injury, greater in the media compared to the intima, which suggests that IGF-1 promotes early medial VSMC proliferation.

Furthermore, the pro-inflammatory cytokines tumour necrosis factor- alpha (TNF-\(\alpha\)), and interleukin- 1\(\beta\) (IL-1\(\beta\)), are upregulated after arterial injury and have been shown to induce intimal VSMC proliferation\textsuperscript{40}, possibly through upregulation of PDGF from endothelial cells.

As previously mentioned, apoptosis is an integral part of the response to arterial injury. Immediately following arterial injury extensive medial VSMC death occurs initiated by medial VSMC stretching and growth factors secreted by VSMCs when injury breaks cell-ECM interactions (30 minutes to four hours\textsuperscript{41} after injury). The remaining VSMCs proliferate and migrate to the neointima. Repair of the vessel after injury is also associated with apoptosis in both the medial and intimal layers and occurs between seven and 21 days after arterial injury in
the carotid artery and aorta\textsuperscript{42}. Several mediators that promote VSMC proliferation also stimulate apoptosis, including oxidative stress (ROS) and cytokines secreted by macrophages (TNF\textgreek{a} and interleukin-6 (IL-6)) and T-lymphocytes (interferon-\gamma)\textsuperscript{43}. These mediators stimulate proto-oncogenes like the Bcl-2 proteins that regulate VSMC apoptosis\textsuperscript{44}. Activation of pro-apoptotic proteins (Bcl-x\textsubscript{s}, Bad, Bid, Bax, Back) induces their translocation to the mitochondria where they release mitochondrial mediators of apoptosis such as caspases\textsuperscript{44}. The overall effect of apoptosis may be beneficial since it balances VSMC proliferation and subsequent neointimal formation.

1.5.5 Vascular Smooth Muscle Cell Migration

As previously mentioned, the subpopulation of VSMCs that escape growth inhibition continue to replicate and migrate from the medial layer across the internal elastic lamina to the luminal surface. This event reaches maximum at four days after arterial injury\textsuperscript{45}. These cells colonize the intimal surface and begin to form a new layer called the neointima. The movement of VSMCs to the luminal surface follows a gradient of chemoattractants in the wall, depends upon the production of matrix degrading proteases, and requires the synthesis of new ECM proteins.

Migration of VSMCs involves a dominant plasma membrane leading edge protruding in contact with an extracellular substrate, and binding integrin transmembrane receptors to form focal complexes and secure focal adhesions\textsuperscript{46}. The cell is propelled forward by actin filament alignment and myosin contraction, as well as the disengagement of the focal adhesions from the cell surface\textsuperscript{47}.

The release of growth factors and ECM proteins serve as potent chemoattractants and enhance VSMC migration. PDGF is the most potent chemoattractant for VSMCs and the PDGF ligand and receptor mRNA are upregulated in the neointima after arterial injury\textsuperscript{48}. PDGF-BB infusion in rats has been shown to increase neointimal growth after arterial injury mainly through
an increase in VSMC migration. In addition, IGF-1 is secreted by VSMCs and endothelial cells in vitro and has also been reported to stimulate migration of human VSMCs.

The ECM is also involved in the VSMC migratory response to vascular injury. Acute arterial injury increases the expression of ECM components including collagen, hyaluronic acid, and glycoproteins. Studies have shown that type VIII collagen regulates VSMC migration after arterial injury by promoting VSMC attachment and chemotaxis. Hyaluronic acid levels are also upregulated in the neointima after injury and have been associated with both endothelial and VSMC migration. The glycoproteins fibronectin, vitronectin, and thrombospondin-1 are upregulated in response to injury and are associated with VSMC migration. The glycoproteins fibronectin, vitronectin, and thrombospondin-1 are upregulated in response to injury and are associated with VSMC migration. Fibronectin has many functions, which include VSMC migration, cellular attachment, and wound healing, and is locally elevated after vessel injury. Osteopontin is another glycoprotein upregulated after injury. It has been shown that neutralizing antibodies against osteopontin decrease neointimal growth after arterial injury. Osteopontin also stimulates matrix metalloproteinase (MMP) activity in VSMCs. Tenascin, another ECM protein, is also increased after arterial injury and regulates cell adhesion and migration.

ECM degrading enzymes also play a role in VSMC migration. For example, elastase activity is increased after arterial injury in rabbits, which could promote greater ECM turnover and facilitate VSMC migration. Cathepsins S and K, proteases that express potent elastolytic and collagenolytic activities, are also increased after arterial injury.

PDGF activates the enzyme calcium/calmodulin-dependent protein kinase II (CamKinaseII), which affects actin filament formation and interaction with small GTP-binding proteins to stimulate VSMC migration. Activation of CamKinaseII in growth-arrested VSMCs restores the ability of these cells to migrate in response to PDGF. Furthermore, calcium channel blockers, amlodipine for example, inhibit VSMC migration, demonstrating that calcium is required for VSMC migration, as low calcium levels inhibit CamKinaseII activation.
induced VSMC adhesion and migration is MAPK-dependent. Furthermore, PDGF has also been shown to activate PI3K to stimulate Rac to promote VSMC migration, which may be upstream of MAPK. PDGF-induced VSMC migration is also partially dependent on integrin receptors, suggesting potential interaction with ECM proteins to further promote VSMC migration.

Small GTPases, including Ras, Rho, Cdc42, and Rac, are also involved in VSMC migration. The GTPases are activated by soluble factors via tyrosine kinase and G protein-coupled receptors and by cell adhesion. Ras activates the MAPK enzyme, extracellular regulated kinase 1/2 (ERK1/2), by activating Raf and MAPK kinase. Furthermore, MAPKs phosphorylate myosin-like light chain kinases to promote cell movement and migration. Rho is also a GTP-binding protein and is involved in mediating cytoplasmic actin filament organization that occurs in migrating cells stimulated by chemoattractants. Rho promotes formation of actin stress fibers and focal adhesions with integrin clusters, and inhibition of Rho has been shown to attenuate VSMC migration. Cdc42 promotes actin polymerization, through Rac activation, to form leading edge lamellipodia and membrane ruffles to promote cell migration. Cdc42 and Rac also activate JNK1/2 and p38 MAPK. Doanes et al. demonstrated that Rac is required for PDGF-stimulated VSMC migration.

NFκB is also activated by arterial injury and is linked to the inflammatory response (upregulates VCAM-1 and MCP-1 expression) associated with increased VSMC migration and neointimal formation. As mentioned in the previous paragraph, PI3K, a member of the insulin signalling pathway, is involved in PDGF-induced VSMC migration as studies suggest that PI3K is necessary for PDGF-induced activation of Rac. PI3K does not appear to be required for cell adhesion but is required for cell spreading and migration. PI3K may also be involved in regulating ECM protein-induced VSMC migration. In addition, Akt phosphorylation and subsequent mTOR/S6K1 activation correlate with increased VSMC migration. For example,
naringin, a compound found in citrus fruit, decreased TNFα- induced PI3K/Akt/mTOR/S6K1 signalling and VSMC migration\textsuperscript{77}. Furthermore, rapamycin, an mTOR inhibitor, decreases VSMC migration\textsuperscript{78}, supporting the role of mTOR in VSMC migration.

1.5.6 Matrix Metalloproteinases (MMPs)

MMPs, zinc- dependent endopeptidases produced by VSMCs, macrophages, and lymphocytes, are involved in ECM degradation, which is necessary for VSMC migration from the medial layer to the intima\textsuperscript{79}. There are four types of MMPs: 1) interstitial collagenases (substrate: interstitial collagen), 2) stromelysins (substrates: laminin and fibronectin), 3) gelatinases (substrate: type IV collagen and gelatin), and 4) membrane type-MMPs (MT-MMPs). The proteolytic activities of MMPs are regulated at three levels: 1) gene expression and synthesis of the inactive pro- enzyme MMP, 2) activation of the pro- enzyme by tissue/ plasma proteinases like plasmin, cell- associated MT- MMPs and by oxidative stress, and 3) inhibition by endogenous tissue inhibitors of metalloproteinases (TIMPs 1- 4) (reviewed in\textsuperscript{79}).

MMPs enable VSMC migration after carotid balloon injury in rats\textsuperscript{45}. The role of MMPs in VSMC migration has been demonstrated by studies where VSMC migration was inhibited by administration of nonselective MMP inhibitors\textsuperscript{45, 80} and by gene transfection of TIMPs into balloon injured vessels in rats\textsuperscript{81}. Various proteinases are induced following balloon injury in various animal models, however in rats mainly MMP- 2 activity and MMP- 9 expression and activity are increased\textsuperscript{82}. Increased MMP- 2 and MMP- 9 activity is also associated with VSMC migration\textsuperscript{82}.

MMPs are secreted in a pro- enzyme form and are activated by limited proteolysis\textsuperscript{83}. Total MMP activity is mainly determined by MMP expression but is also regulated independently by TIMPs and ROS\textsuperscript{79}. MMP expression may be regulated by ECM proteins, cytokines (IL- 1 and TNFα), and growth factors (bFGF, PDGF, TGFβ, and EGF)\textsuperscript{84}. Several of these activate transcription factors such as activating protein- 1 (AP- 1) (through MAPK
signalling) and nuclear factor kappa B (NFκB) that regulate MMP expression. Type VIII collagen, a matrix protein that is upregulated in the rat carotid artery after balloon injury, has been shown to bind to integrin receptors on VSMCs to stimulate MMP synthesis and VSMC migration. For example, αvβ3 integrin receptors are upregulated in VSMCs following balloon injury and mediate VSMC migration by stimulating MMP (MMP- 2 and MMP- 9) synthesis. Conversely, nitric oxide (NO) decreases MMP- 2 and MMP- 9 expression and also increases TIMP-2 production to inhibit VSMC migration, perhaps as a result of inhibition of the NFκB signalling cascade.

1.5.7 Extracellular Matrix

Normally, the ECM acts as a barrier to VSMC migration from the media to the intima, however after arterial injury it is modified to allow the movement of cells. The ECM constitutes the majority of the neointima as cellular components make up only approximately 11%. The ECM in the artery is composed of the scaffolding elements of collagens (types I, III, IV, and VI) and elastin embedded in a mixture of glycoproteins (e.g. fibronectin) and proteoglycans (e.g. heparan sulphate). Both collagen and elastin are synthesized by endothelial cells, intimal and medial VSMCs, and adventitial fibroblasts.

Collagens make up the major structural component of ECM in the artery and their expression is increased after balloon injury. Collagen exists as either a meshwork surrounding individual VSMCs or as interstitial dense fibers that occupy a large volume of the tissue. Elastin is one of the most abundant proteins of large arteries and provides elasticity. The synthesis of the ECM proteins collagen (type VIII collagen in particular) and elastin is increased after arterial balloon injury reaching significant elevation at 21 and 60 days after injury, despite the increased production of MMPs, plasminogen activators and plasmin that degrade matrix proteins. This may be due to a transient induction of proteases that are mainly involved in VSMC migration and not ECM degradation. Increases in elastin synthesis
appears to be more significant than collagen synthesis, which may be explained by elastin’s
sensitivity to mechanical forces of blood flow or balloon expansion attributed to the greater
distensibility of elastin. The ECM interacts with cells via cell surface receptors like integrins,
which are upregulated after balloon injury in rats. Binding of collagen to integrins initiates
intracellular signalling cascades by recruiting cytoplasmic tyrosine kinases and adapter proteins
to focal adhesion sites at the cell membrane. As previously discussed, dynamic adhesive
interactions between cell surface integrins and the ECM play a central role in VSMC migration
after balloon injury by providing cell-substrate contact and cell motility.

Re-expression of the embryonic forms of fibronectin occurs in the media and adventitia
of rabbit arteries 24 to 48 hours after injury; two weeks after balloon injury when the neointima
is formed, fibronectin mRNA and protein accumulates in the luminal layers of the neointima.
Osteopontin is also elevated after arterial injury and as previously mentioned, neutralizing
antibodies against osteopontin decrease neointimal growth after injury. TGF-β1 increases the
synthesis of fibronectin, fibrillar collagens, elastin, thrombospondin, and proteoglycans, all of
which are increased after arterial injury.

1.5.8 Endothelial Regeneration

Arterial balloon angioplasty results in complete endothelial denudation, however soon
after injury neighbouring endothelial cells begin to replicate in response to growth factors such
as VEGF and bFGF, to restore the protective endothelial layer and prevent neointimal
formation. VEGF is well known to stimulate endothelial cells, however it does have effects on
monocytes, macrophages, hematopoietic stem cells, and VSMCs (reviewed in). The mitogenic
action of VEGF primarily affects endothelial cells, thus providing an advantage of VEGF
application in the vessel. Phosphorylation of endothelial nitric oxide synthase (eNOS) at Serine
1177 by Akt is required for VEGF-induced endothelial cell migration. Furthermore, VEGF
binding to VEGFR2 has been shown to initiate autophosphorylation of the receptor and lead to
Grb2 activation through Src binding. This leads to ERK1/2 and p38 MAPK activation, which is involved in endothelial cell proliferation and migration respectively\textsuperscript{100}. Despite this, the role of VEGF in reducing neointimal formation remains unclear as VEGF treatment was also shown to promote restenosis\textsuperscript{101}. In addition, a clinical trial of local VEGF delivery for restenosis did not show any benefit\textsuperscript{102}.

Regeneration of the endothelium begins from the leading edge of the denuded area within 24 hours of arterial injury and ceases approximately six weeks later\textsuperscript{103}, however, depending on the extent of injury, it is seldom complete in the carotid artery\textsuperscript{104}. If the denuded area is a relatively short distance between adjacent sources of endothelium, more complete endothelial regeneration occurs\textsuperscript{104}. Neointimal thickness is decreased by this re-endothelialization process as the endothelial layer decreases the proliferation of underlying VSMCs through NO production\textsuperscript{104}, which is discussed further in section 1.7. Therefore, delays in re-endothelialization result in greater neointimal growth. \textit{In vivo}, administration of L-arginine, which is catalyzed by NOS to release NO, prevents neointimal growth\textsuperscript{105}. eNOS transfection into the vessel wall after balloon injury in rats increased local NO production and decreased VSMC proliferation, migration, and ECM production, which are involved in neointimal formation\textsuperscript{106}. Additionally, in bovine and porcine models of carotid injury, perivascular implantation of endothelial cell grafts reduced neointimal growth by increasing NO availability\textsuperscript{107}. Neointimal growth is also increased in eNOS knockout mice after carotid ligation despite an intact endothelium\textsuperscript{108}. These studies provide evidence that restoration of the endothelium and increased NO availability are important for limiting neointimal formation after arterial injury.

1.5.9 Progenitor Cell Contribution

Stem cells are capable of self-renewal and differentiation while progenitor cells are more differentiated. Together they constitute approximately 0.1% of total bone marrow cells in humans. Stem cells have been broadly classified as either hematopoietic (HSC) or mesenchymal
(non-hematopoietic) (MSC) stem cells. HSCs differentiate into hematopoietic progenitor cells and then into various blood cell lines, whereas MSCs differentiate into adipocytes, chondrocytes, osteoblasts, and muscle cells (reviewed in 109). Endothelial progenitor cells (EPC), a subset of bone marrow-derived progenitor cells, are thought to have the same precursor as hematopoietic stem cells 110. In addition to the progenitor cells residing in the bone marrow, there is a small population of progenitor cells maintained in the circulation 111, which can differentiate into either endothelial or VSMCs.

There is evidence in studies using bone marrow transplant in combination with mouse 112 or rat 18 models of vessel injury, that several types of progenitor cells are also a source of newly differentiated VSMCs and increase neointimal formation. However, other studies in mice suggest that the new population of VSMCs at the site of vessel injury originate as either progenitor cells that permanently reside in the vessel wall 113 or as VSMCs resident in the vessel wall 19 and not from bone marrow-derived progenitor cells. The controversy is a result of the methodology chosen to identify the cells as being double-positive for a bone marrow-derived cell and VSMC. Markers for VSMCs (SMα-actin in particular) can be expressed by other cell lineages and also the low resolution microscopy used in some studies can lead to false positive identification of cells (reviewed in 114).

As mentioned in the previous section, restoration of the endothelium decreases neointimal formation and it was previously thought that the endothelial cells at the perimeter of the denuded area were the primary source of endothelial cell replication, however re-endothelialization may also occur from circulating or bone marrow-derived progenitor cells. Recent studies have shown that circulating and bone marrow-derived EPC recruitment accelerates re-endothelialization and decreases neointimal formation after vessel injury 20. Furthermore, Fadini et al 115 demonstrated that diabetes impairs EPC mobilization after
ischaemia-induced injury in rats and that lowering of hyperglycemia with insulin partially recovered EPC mobilization.

The majority of studies were performed in mice and the progenitor cell contribution to neointimal growth appears to vary by the type of vessel injury\textsuperscript{116}. Tanaka et al\textsuperscript{116} showed that mechanical injury using a straight wire to denude and dilate the mouse femoral artery resulted in the greatest progenitor cell recruitment to the arterial wall compared to a polyethylene tube placed around the femoral artery or ligation of the common carotid artery proximal to the bifurcation.

Bone marrow-derived progenitor cell recruitment after arterial injury to the neointima occurs in three stages; 1) cell mobilization from the bone marrow, 2) bone marrow cell migration and recruitment to the site of injury, and 3) bone marrow cell differentiation into vascular cells. These stages are closely related as several signalling molecules are involved in multiple stages. Studies suggest that bone marrow progenitor cells are mobilized to the peripheral circulation in response to injury-induced stress signals\textsuperscript{117}. Exit from the bone marrow, which is rich in stromal cells and ECM proteins that attach to the progenitor cells through integrin interaction, requires migration of progenitor cells through a vascular barrier that separates the hematopoietic compartment from the circulation\textsuperscript{117}. Granulocyte Colony Stimulating Factor (G-CSF) stimulates progenitor cell mobilization\textsuperscript{118} by increasing proteases to cleave adhesion molecules. G-CSF also stimulates MMP-9, which releases soluble c-kit and c-kit ligand (KitL) (also known as Stem Cell Factor) so that c-kit positive progenitor cells can be recruited\textsuperscript{119}. Similarly, stromal derived factor-1α (SDF-1α) also increases progenitor cell mobilization in the bone marrow by activating MMP-9, which subsequently cleaves KitL\textsuperscript{119}.

Once in the peripheral circulation, progenitor cells migrate to the site of injury. SDF-1α stimulates progenitor cell recruitment by increasing platelet adhesion to the site of injury and
stimulating platelet p-selectin to promote progenitor cell adhesion and recruitment\textsuperscript{120}. VEGF/VEGFR also increase progenitor cell migration and recruitment to the site of injury\textsuperscript{101}.

At the site of arterial injury, bone marrow-derived progenitor cells differentiate into endothelial cells or VSMCs to integrate into the neointima. VEGF promotes endothelial cell differentiation\textsuperscript{121}, whereas TGF-β and PDGF-BB have been demonstrated to induce differentiation into VSMCs\textsuperscript{122}. Direct cell-to-cell contact may also stimulate differentiation\textsuperscript{122}.

The origin and contribution of progenitor cells to the vessel response to injury is very controversial as much of our understanding is from a small number of animal studies. Even less is known regarding the specific contribution of progenitor cells to vascular disease in humans, however stem cell therapy for vascular disease is currently being tested in clinical trials\textsuperscript{123}.

### 1.6 Type 2 Diabetes and Metabolic Syndrome

Type 2 diabetes has become a worldwide epidemic where macrovascular complications are the major cause of mortality in diabetic patients\textsuperscript{3}. Type 2 diabetes is characterized by insulin resistance, defined as the inability of insulin to exert its metabolic actions, and a defect in insulin secretion from the pancreatic β cells, which is inadequate to compensate for insulin resistance\textsuperscript{3}. More than 80% of type 2 diabetic subjects are obese and obese persons often have elevated plasma levels of free fatty acids (FFAs) due to their expanded and more lipolytically active adipose tissue stores. It is now well established that insulin resistance itself is a potent risk factor for atherosclerosis and restenosis after percutaneous interventions\textsuperscript{124}, and the risk increases when hyperglycemia is present\textsuperscript{125}. As a result, diabetic patients account for approximately one quarter of coronary revascularization procedures\textsuperscript{126} and have a greater restenosis rate than non-diabetic subjects after percutaneous interventions such as angioplasty and stenting and require repeat revascularization\textsuperscript{127}. Insulin resistance is a typical feature of a cluster of risk factors for both atherosclerosis and type 2 diabetes, which has been termed the metabolic syndrome.
The metabolic syndrome affects approximately 25% of the population and is characterized by a cluster of abnormalities which include central obesity, insulin resistance, compensatory hyperinsulinemia, dyslipidemia, and hypertension and confer a high risk for type 2 diabetes and atherosclerosis. Currently, the most accepted hypothesis to describe the pathophysiology of metabolic syndrome is insulin resistance, which can be caused by obesity via the release of fatty acids and other secretory products such as cytokines from the expanded fat tissue. Hyperinsulinemia is generally considered to be secondary to insulin resistance but may also be stimulated by excess circulating nutrients. Furthermore, excess circulating insulin can aggravate insulin resistance, contributing to a vicious cycle. The metabolic and cardiovascular features of metabolic syndrome (i.e. dyslipidemia, hypertension) greatly contribute to atherosclerosis; however there are vascular effects of insulin by itself, which are both atherogenic and vasculoprotective. The atherogenic effect of insulin appears to be MAPK-dependent while the vasculoprotective effect appears to involve the PI3K pathway. Insulin has mostly protective effects in the endothelium and platelets, which are present in the intact vessel, but are absent in VSMC cultures. Therefore, the prevailing effect of insulin on atherogenesis in vivo is now considered to be protective and instead, insulin resistance is considered to be harmful, because of the unresponsiveness to insulin action, including its vascular anti-inflammatory properties. Studies from our laboratory support a protective role of insulin against restenosis and will be discussed further in this thesis.

The obesity-associated pro-inflammatory state due to cytokine release from adipose tissue and to ROS generation by excess nutrients has recently been suggested to be the major cause of insulin resistance. Oxidative stress and associated inflammatory damage are also mediators of vascular injury in atherosclerosis and restenosis. Therefore, it is possible that in obesity, both insulin resistance and atherosclerosis are caused, at least in part, by inflammation.
1.7 Nitric Oxide

Nitric oxide (NO) plays a pivotal role in the regulation of vascular homeostasis. NO is produced through the conversion of L-arginine to L-citrulline by NOS and exerts its effects by activating guanylate cyclase, which increases cyclic guanylate monophosphate (cGMP) formation, and then activates protein kinase G (PKG). NO bioavailability in the vasculature is greatly influenced by the presence of superoxide. Superoxide degrades NO and forms peroxynitrite, which plays a role in the pathogenesis of several diseases.

The family of NOS enzymes consists of three members: eNOS, inducible NOS (iNOS), and neuronal NOS (nNOS). Although these isoforms are very different, they all require the cofactors nicotinamide adenine dinucleotide phosphate hydrogen (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin (BH$_4$) to catalyze the enzyme reaction. eNOS and nNOS are constitutively present in many tissues and NO production is mainly regulated by intracellular calcium fluxes that permit calmodulin binding, which activates the enzyme. eNOS gene expression can also be up- or down-regulated by various stimuli and regulated through phosphorylation of the enzyme. eNOS can be phosphorylated on multiple serine, threonine, and tyrosine residues, however Serine1177 is the major positive regulatory site and is phosphorylated by Akt (activated by insulin, VEGF) and 5' adenosine monophosphate-activated protein kinase (AMPK) (activated by resveratrol, metformin). In contrast, iNOS is not normally produced by most cells as it is transcriptionally regulated and is induced by cytokines. NO output differs between each NOS isoform where eNOS produces the least and iNOS produces the greatest amount of NO.

Reduced bioavailability of the main substrate L-arginine or depletion of the NOS cofactor BH$_4$ by peroxynitrite-induced oxidation can compromise NOS function and result in “uncoupling” of the NOS dimer. The term “NOS uncoupling” has been used to describe this
event whereby electron flow through the enzyme in interrupted and superoxide is produced instead of NO. As previously mentioned, superoxide can then react with NO to form peroxynitrite, thus promoting a vicious cycle to further deplete NO. In general, NOS uncoupling is detrimental to the vasculature as it results in both an increase in ROS production and loss of the protective effects of NO.

In the rat arterial balloon injury model, the main sources of NO are endothelial cells (in an injured vessel there are endothelial cells along the perimeter of the de-endothelialized area\textsuperscript{146}), circulating EPCs\textsuperscript{20}, platelets\textsuperscript{147} (all of which express eNOS), and VSMCs (express iNOS and nNOS)\textsuperscript{148}. It is well established that NO diminishes neointimal growth after injury\textsuperscript{105, 149}, whereas NOS inhibition aggravates restenosis\textsuperscript{150}. Gene transfer studies have shown that upregulation of eNOS diminishes neointimal formation after arterial injury in rats\textsuperscript{151, 152}. In turn, eNOS deficiency results in greater neointimal growth after cuff injury in mice\textsuperscript{153}. The primary role of iNOS on neointimal formation is controversial, as studies in knockout mice indicate that iNOS accelerates restenosis\textsuperscript{154}, whereas several gene transfer studies indicate that it decreases it\textsuperscript{155-157}. Based on the latter studies, the prevalent view is that iNOS induction after injury is beneficial, although its effect may be variable because iNOS can produce superoxide when substrates and cofactors for synthesis of NOS are lacking\textsuperscript{158}. There is one report of induction of nNOS in VSMC after arterial injury and aggravation of neointimal formation after nNOS inhibition\textsuperscript{159}, however another study reported that nNOS was not present in vessel cross-sections before or after arterial injury in rabbits\textsuperscript{160}.

Consistent results have yet to be demonstrated in humans, although numerous animal studies have demonstrated a protective effect of NO on restenosis after vessel injury. In the Angioplastique Coronare Corvasal Diltiazem study, patients that underwent angioplasty and received NO from i.v. linsidomine followed by oral molsidomine for six months had a decreased occurrence of restenosis\textsuperscript{161}. However in a separate study, there was no effect of NO in patients
that received high-dose oral molsidomine for six months after angioplasty\textsuperscript{162}. In addition, the delivery method of NO is limiting as systemic administration can cause hypotension, headaches, and increased bleeding. This highlights an advantage of treatment with compounds such as insulin or RSV that may also stimulate NO production to inhibit restenosis and could be administered locally (i.e., in a drug-eluting stent).

NO acts through several mechanisms to protect the vasculature. Specifically, NO induces vasorelaxation, and inhibits several of the processes associated with atherosclerosis development following endothelial cell damage, including leukocyte-endothelial adhesion, VSMC migration and proliferation, as well as platelet aggregation (reviewed in \textsuperscript{163}). In the rat arterial balloon injury model NO inhibits platelet aggregation and adhesion, and increases blood flow\textsuperscript{164}. Furthermore, in the same study iNOS expression was increased in the innermost layer of the media and in the neointima, suggesting that iNOS may be a major source of NO when the endothelium is not present. NO has also been shown to inhibit leukocyte accumulation as an increase in adherent leukocytes was found in iNOS knockout mice\textsuperscript{165}.

As discussed in section 1.5.8, the effect of NO on the endothelial layer is protective, as NO stimulates endothelial regeneration after vessel injury in the rat\textsuperscript{166}. It appears that VEGF, when added to coronary endothelial cells in culture, increases cytoplasmic calcium levels to stimulate eNOS and NO production and subsequently cGMP, resulting in MAPK activation and increased cell proliferation\textsuperscript{167}. In this model, the mechanism of cGMP to promote MAPK activation was not examined. Furthermore, in eNOS knockout mice, the angiogenic response (which requires endothelial cell proliferation) to hindlimb ischemia was diminished and could not be stimulated by VEGF\textsuperscript{168}. NO also suppresses endothelial cell apoptosis to maintain the integrity of the endothelium. For example, cultured endothelial cells subjected to shear stress\textsuperscript{169} or lipopolysaccharide\textsuperscript{170} did not undergo apoptosis in the presence of increased NO.
In contrast to endothelial cells, studies have shown that NO plays a role in decreasing VSMC proliferation and the mechanism(s) are more clear\textsuperscript{171, 172}. NO inhibits the G1/S phase transition to induce cell cycle arrest by upregulating the expression/activity of cell cycle regulatory proteins like p21 or cyclin-dependent kinase \textsuperscript{2173}. NO also activates cGMP and PKG to decrease VSMC growth, as NO donors inhibit DNA synthesis in VSMCs\textsuperscript{171}. This effect involves PKG to decrease intracellular calcium levels by inhibiting calcium release from the sarcoplasmic reticulum, decreasing calcium influx across the plasma membrane, and promoting calcium efflux. The decrease in intracellular calcium reduces calcineurin activation and thus inhibits proliferation\textsuperscript{174}. Furthermore, the growth inhibitory effect of NO, possibly from endothelial cells, may maintain VSMC quiescence\textsuperscript{171}. Xu et al\textsuperscript{175} demonstrated that endothelial cells stimulate thioredoxin, an endogenous anti-oxidant, through a NOS- and NADPH oxidase-independent mechanism to decrease ROS in VSMCs and inhibit VSMC proliferation. Studies also indicate that NO induces apoptosis in rabbit VSMCs\textsuperscript{176}. iNOS overexpression in transfected VSMCs promoted apoptosis, whereas L-NMMA, a NOS inhibitor, decreased apoptosis in iNOS-transfected VSMCs\textsuperscript{177}. This increase in NO production by iNOS was associated with an increase in the tumour suppressor gene p53, which induces apoptosis in many cell types. NO also inhibits VSMC migration, as NO donors inhibited the number and distance of migrating VSMCs\textsuperscript{178} and also angiotensin-II-induced VSMC migration measured \textit{in vitro}\textsuperscript{179}.

As previously discussed, migration of VSMCs depends upon growth factor-induced increases in cytosolic calcium, which may be inhibited by NO by preventing the increase in calcium\textsuperscript{180}. Furthermore, VSMC migration is prevented by the ECM barrier, whereas MMPs degrade the basement membrane and ECM to allow VSMC migration. eNOS transfection into rat VSMCs decreased MMP-2 and MMP-9 activity and increased TIMP-2 activity\textsuperscript{181}, suggesting that NO may inhibit VSMC migration in part by regulating MMPs. NO also affects matrix elements. L-arginine, the substrate for NO production, decreased hyaluronan synthase
expression. There are contradictory reported effects concerning the effect of NO on ECM proteins. NO has been shown to inhibit basal type I collagen levels and collagen synthesis in vitro whereas NO donors increased collagen I and III synthesis in human coronary VSMCs. Furthermore, NO treatment decreased osteopontin expression in human saphenous vein segments ex vivo. Taken together, these studies suggest that NO has a protective effect to prevent neointimal hyperplasia after vessel injury as it inhibits several key processes, including VSMC migration and proliferation and ECM production, involved in the formation of the intima.

1.8 Insulin

1.8.1 General effects of insulin.

Insulin action results in a variety of biological outcomes, including control of glucose, fat, and protein metabolism, cellular growth and development. In the liver, insulin increases glycogen synthesis and suppresses hepatic glucose production. Insulin promotes energy storage in adipocytes by increasing glucose and FFA uptake, and stimulating lipogenesis while inhibiting lipolysis. In skeletal muscle insulin stimulates glucose and amino acid uptake and promotes glycogen and protein synthesis.

The role of insulin in metabolism is well defined and established; however the effect of insulin in the vasculature has only more recently been considered and is less clear. As previously mentioned, it is debated whether insulin is protective or detrimental in the vasculature. Protective effects of insulin include stimulation of NO production (through PI3K activation) and subsequent vasodilation, inhibition of platelet adhesion and aggregation, and anti-inflammatory effects, whereas the detrimental effects include growth-promotion (through MAPK activation) and vasoconstriction.
1.8.2 Effects of insulin on atherosclerosis.

Initially, insulin was thought to promote atherosclerosis, based on epidemiological studies which showed an association between hyperinsulinemia and atherosclerotic CVD\textsuperscript{188, 189}. However, this concept has been challenged as hyperinsulinemia is often accompanied by other risk factors such as dyslipidemia and hypertension\textsuperscript{190} and usually coexists with (and is a marker of) insulin resistance. Epidemiological studies have described an association of atherosclerotic CVD and insulin resistance\textsuperscript{124, 191}. In the Insulin Resistance Atherosclerosis Study (IRAS)\textsuperscript{124}, the association was greater than that with hyperinsulinemia. The Diabetes Control and Complications Trial (DCCT)\textsuperscript{192} and United Kingdom Prospective Diabetes Study (UKPDS)\textsuperscript{193} studies have demonstrated that intensive treatment of diabetes to increase insulin levels or action does not increase but actually decreases atherosclerotic CVD. These results are likely due to the effect of insulin to improve glycemic control, nevertheless they suggest that insulin is, at least, not detrimental.

Whether insulin is atherogenic or vasculoprotective has not been adequately assessed in animal models of atherosclerosis, in part due to the limitations of the non-transgenic models. Early studies showed that insulin treatment aggravated atherosclerosis in cholesterol-fed alloxan-diabetic\textsuperscript{194} and non-diabetic rabbits\textsuperscript{195}, and delayed the regression of atherosclerosis in cholesterol-fed chickens re-fed a normal diet\textsuperscript{196}. In contrast, in later studies insulin treatment did not promote atherogenesis in cholesterol-fed rabbits\textsuperscript{197}. Instead, massive doses of oral insulin absorbed systemically protected against atherogenesis (decreased both size and number of lesions) in Apolipoprotein E (ApoE) knockout mice\textsuperscript{198} (ApoE knockout mice develop spontaneous atherosclerotic lesions without high-fat feeding), whereas systemic knockout\textsuperscript{199, 200} and disruption of only one allele\textsuperscript{201} of insulin receptor substrate-2 (IRS-2) aggravated atherogenesis in the same model compared to the ApoE knockout mice with intact IRS-2. In addition to the species differences, the discrepancy over whether insulin is protective or
atherogenic may be explained by the different diets used in these studies. For example, in the rabbit and chicken studies high-cholesterol diets were used, whereas either normal chow or high-fat diets were used in the studies with mice. Both high-cholesterol and high-fat diets are known to induce insulin resistance, which was not measured in the studies mentioned, and in the insulin resistant state VLDL production by the liver is increased (reviewed in 202). Insulin is lipogenic and normally has an acute suppressive effect on VLDL production from the liver. However, it is not clear whether in conditions of insulin resistance the increase in VLDL production is due to insulin resistance or hyperinsulinemia per se (reviewed in 202). Therefore, it is possible that high-cholesterol feeding in combination with insulin treatment may have increased lipid deposition in the vessel wall resulting in promotion of atherosclerosis.

The latter findings can be taken as evidence for a protective effect of insulin signalling, unless compensatory and possibly atherogenic pathways (e.g. Shc, IRS-1) were potentiated. In the mouse, complete disruption of IRS-2 also results in systemic effects which in turn can influence atherogenesis, such as hypertension and hyperglycemia, in addition to insulin resistance and hyperinsulinemia 203 and similarly in doubly deficient mice (ApoE/IRS-2-null) increased glucose intolerance, hyperinsulinemia, and insulin resistance are present 200. Partial disruption of IRS-2 (single allele) in ApoE-null mice fed standard chow aggravates atherosclerosis but does not affect body weight, circulating glucose levels, fasting plasma insulin levels, glucose tolerance or glucose-stimulated insulin secretion, however when fed a high-fat diet, heterozygous IRS-2 null mice show increased fasting plasma insulin levels and increased glucose intolerance 201. Although systemic IRS-2 knockout aggravates atherosclerosis, the effect of selective disruption in insulin signaling is controversial. Recent studies have reported opposite results on atherosclerosis with selective knockout of insulin receptor or IRS-2 in myeloid cells in low-density lipoprotein receptor (LDLR) knockout 204 (LDLR knockout mice develop atherosclerotic lesions with high-fat feeding) and ApoE knockout mice 205, thus rekindling the
debate about the atherogenic vs. vasculoprotective effects of insulin. However, preliminary studies in ApoE- null mice with selective endothelial knockout of insulin receptor indicate that the action of insulin in endothelium is indeed protective\textsuperscript{206}.

IGF-1 shares similar signaling with that of insulin but preferentially stimulates MAPK versus PI3K and along with other growth factors like PDGF, traditionally has been considered to promote the development of atherosclerosis by stimulating VSMC migration and proliferation\textsuperscript{207}. Furthermore, its expression was found to be increased in aortic lesions of ApoE knockout mice compared to non-lesion areas\textsuperscript{208}. However, a recent study reported that low-dose IGF-1 decreased plaque burden in ApoE knockout mice while increasing phosphorylated Akt, eNOS, and circulating EPCs\textsuperscript{209}, suggesting a beneficial role of IGF-1 in atherosclerosis, similar to the role of insulin.

1.8.3 Effects of insulin on restenosis.

A few studies have evaluated the effect of insulin on the arterial response to injury. Some studies suggest that insulin treatment aggravates intimal thickening in models of type 1 diabetes, perhaps by increasing IGF-1\textsuperscript{210, 211}. In models of insulin resistance, such as the Zucker Fatty rat\textsuperscript{212}, the fructose-fed rat\textsuperscript{213}, and the Zucker diabetic fatty (ZDF) rat\textsuperscript{214}, neointimal growth after injury is increased, however it is not clear whether this is due to insulin resistance or to hyperinsulinemia. Insulin treatment of non-diabetic rats did not affect DNA synthesis in injured aortas during normoglycemia or hyperglycemia but decreased DNA synthesis in the presence of hypoglycemia\textsuperscript{215}, however, intimal area was not measured. Prior to our studies, there were only a few reports on the effect of insulin on intimal thickness after balloon injury in non-diabetic models and their results were opposite: in one study by Indolfi et al\textsuperscript{216}, insulin treatment via islet transplantation increased neointimal growth, whereas in another study by Kubota et al\textsuperscript{217} neointimal growth after cuff-injury was greater in mice with a defect in insulin signalling (IRS-2 null mice). Thus the effect of insulin on restenosis remains a controversial issue.
A major goal of our laboratory is to examine the effects of insulin on restenosis and explore the potential mechanisms involved. In the initial study by my colleagues, the effect of insulin on vascular growth after balloon catheter injury in rats fed a control (12% fat in calories) or a high-fat diet (60% fat) was determined\textsuperscript{133}. Plasma insulin levels were elevated (levels equivalent to those seen in insulin resistant Zucker Fatty rats) by s.c. insulin implants (5U/day in a 425-450g rat) given two days before carotid balloon injury. The insulin-treated groups received glucose in drinking water to avoid frank hypoglycemia (<3.3 mM), however plasma glucose levels declined to the non-hypoglycemic range (~4 mM). Intimal area evaluated 14 days after injury was decreased in insulin-treated rats on the control diet, however the effect of insulin was diminished in the rats on the high-fat diet. Intimal cell proliferation was greater than control in all treated groups. Thus, a mitogenic effect of insulin was observed at this dose, however the predominant effect of insulin was to decrease neointimal growth despite increasing proliferation. These results are the first evidence for a protective effect of subcutaneous insulin treatment in a model of restenosis and are in accordance with those of the previously mentioned mouse study with increased neointimal growth when insulin signaling was disrupted (IRS-2 deficiency)\textsuperscript{217}.

In a subsequent study, we defined the effect of insulin on VSMC kinetics over the time course of neointimal formation\textsuperscript{134}. A lower dose of insulin (3U/day in a 425-450g rat) and i.p. glucose infusion soon after surgery allowed us to investigate the effect of insulin concentrations that can be seen also in non-insulin resistant rats after an oral glucose load, while avoiding glucose-lowering. Insulin decreased neointimal area at its peak (28 days), reduced elastin content and tended to decrease collagen content. Intimal proliferation and apoptosis were not changed by this dose of insulin. However, insulin markedly inhibited cell migration evaluated at its peak (four days after injury).
In preliminary studies we found that insulin inhibits MMP-2 expression and activity and MMP-9 activity, which is evidence for a mechanism whereby insulin could inhibit VSMC migration\textsuperscript{218}.

However, in contrast to our studies, Foster et al\textsuperscript{219} reported that subcutaneous insulin (~4U/day) given by an osmotic pump enhanced neointimal formation after vascular injury in normal rats. Instead of the 40% glucose water used in our studies\textsuperscript{133}, 10% sucrose water was given to maintain normoglycemia, which could have induced some insulin resistance\textsuperscript{220}. Thus, it is possible that the increase in neointimal growth observed when insulin was combined with oral sucrose was due to sucrose and not to insulin, which is addressed in Study 1 of this thesis.

In agreement, Pu et al\textsuperscript{221} has also shown that insulin infusion (~1.6U/day in a 180-200g rat equivalent to ~3.6U/day when compared to a 425-450g rat), using the same s.c. insulin-releasing pellet as in our studies\textsuperscript{133, 134}, increases neointimal formation possibly via suppression of protein tyrosine phosphatase 1B (PTP1B) function leading to enhanced PDGF receptor signaling.

Most recently the insulin analog glargine (equivalent to ~3.5U/day) was shown to reduce neointimal formation in Zucker fatty rats\textsuperscript{222}, which exhibit hypertriglyceridemia, obesity, hyperinsulinemia, and insulin resistance. The attenuation of neointimal formation was associated with a decrease in tissue oxidative stress and an increase in plasma IGF-1 levels. Therefore, it is possible that the beneficial effect of glargine is due to insulin and/or IGF-1. As discussed above, there are suggestions of a dual effect of IGF-1 on atherosclerosis, similar to insulin. IGF-1 is overexpressed in the rat aorta after arterial injury\textsuperscript{38}, however its major effect on restenosis does not seem to be protective. For example, a D-peptide analogue of IGF-1 inhibited VSMC proliferation but did not decrease neointimal formation after carotid balloon injury in the rat\textsuperscript{223}.

In the same model, blocking IGF-1 signalling with a dominant negative IGF-1 receptor
adenovirus decreased neointimal formation\textsuperscript{224}. Subsequent studies in mice have also shown that IGF-1 increased intimal thickness after arterial injury\textsuperscript{225}.

1.9 Mechanism of Insulin Action in the Vasculature

1.9.1 General features of insulin signal transduction pathways.

Insulin signal transduction involves two major pathways- 1) the PI3K signalling pathway (primarily responsible for metabolic functions) and 2) the MAPK signalling pathway (important for cell growth and development) (please see Figure 1.1 for a summary of insulin signalling). Insulin binding to its receptor results in autophosphorylation of tyrosine residues (reviewed in\textsuperscript{226}). The resulting tyrosine kinase activity enables the insulin receptor to phosphorylate the tyrosine residues of IRS-1 or IRS-2 and Shc, which serve as docking proteins for downstream signalling molecules. Phosphorylated IRS is then able to associate with the p85 regulatory subunit of PI3K, which activates the catalytic subunit p110. Once activated, PI3K phosphorylates the substrate phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) to generate the lipid phosphatidylinositol 3,4,5 trisphosphate (PIP\textsubscript{3}), which in turn activates phosphoinositide-dependent protein kinase-1 (PDK-1) and PDK-2. PDK-1 activates Akt (phosphorylates the Threonine 308 residue) and atypical protein kinase C isoforms \( \zeta \) and \( \lambda \) (PKC\( \zeta \) and PKC\( \lambda \)). The role of PDK-2 is not clear and it is suggested that PDK-2 is actually rictor- mTOR (TORC2), a protein complex of mTOR, rictor, and mammalian stress-activated protein kinase interacting protein 1, that regulates the cytoskeleton. “PDK-2” phosphorylates Akt at the Serine 473 residue, a separate site from PDK-1, and it is suggested to mediate mTOR- S6K1 binding (reviewed in\textsuperscript{227}). Akt phosphorylates many substrates including; eNOS to stimulate NO production\textsuperscript{228} and forkhead transcription factor (FOXO1) to inhibit gluconeogenesis. Insulin-induced Akt phosphorylation also activates mTOR to stimulate S6K1 and/or phosphorylate eukaryotic initiation factor-4E binding protein-1 (4E-BP1) to promote protein synthesis.
Akt also phosphorylates the pro-apoptotic Bcl-2 family member Bad to prevent apoptosis.

As previously mentioned, the docking protein Shc interacts with the insulin receptor resulting in phosphorylation of tyrosine residues on Shc, thus enabling it to bind to growth factor receptor-binding protein 2 (Grb2). Grb2 can also bind to IRS-1 for activation. Grb2 brings the pre-associated GTP exchange factor Son of sevenless (Sos) to the proximity of Ras. This puts Ras in the GTP bound form (the activated form), thus enabling Ras to stimulate Raf, which initiates a kinase phosphorylation cascade involving MEK1/2 and ERK1/2 MAPK. MAPK activation regulates growth and mitogenesis through gene transcription and also increases endothelin-1 (ET-1) secretion to induce vasoconstriction. Protein tyrosine phosphatases, such as PTP1B, SHP-1, and SHP-2, dephosphorylate the insulin receptor and IRS-1 and lipid phosphatases, such as SHIP-2 and PTEN, dephosphorylate PIP3 to inhibit insulin signalling.

Very recently, insulin-stimulated NO production was shown to inactivate protein tyrosine phosphatases to enhance insulin responsiveness in endothelial cells.

1.9.2 Insulin stimulates NO production and induces vasodilation.

One of the most important protective vascular effects of insulin is stimulation of eNOS, to increase NO production, via PI3K and Akt in endothelial cells. Insulin has also been reported to upregulate nNOS in neurons, and iNOS in VSMCs. Furthermore, insulin treatment was also shown to augment NO-stimulated guanylate cyclase activity in VSMCs. eNOS has many phosphorylation sites, however it is widely accepted that the Serine 1177 residue (equivalent to Serine 1179 in bovine eNOS) is the major regulatory site of insulin-stimulated eNOS activation. However, a very recent study suggests that eNOS phosphorylation at Serine 615 also contributes to insulin-stimulated NO production. This phosphorylation-dependent mechanism to activate eNOS is calcium-independent. Even though Akt is necessary for insulin-stimulated activation of eNOS, Akt activation alone is not
sufficient for eNOS activation\textsuperscript{235}. A possible explanation for this specificity is that association of heat shock protein 90 (HSP90) with eNOS is required for eNOS-mediated NO production\textsuperscript{244}. Takahashi et al\textsuperscript{244} demonstrated that insulin stimulation causes HSP90-Akt-eNOS association and results in subsequent eNOS phosphorylation, whereas in the absence of HSP90, Akt was not observed in the eNOS complex. The interactions between HSP90, Akt, and eNOS are not clear, however HSP90 may facilitate Akt activation of eNOS by acting as a scaffold to promote kinase-substrate association.

As previously mentioned, insulin does not affect intracellular calcium levels in endothelial cells, suggesting that insulin-stimulated NO production is calcium-independent\textsuperscript{237} and therefore dissimilar to classical vasodilators such as acetylcholine. NO diffuses into the VSMCs and endothelium to promote vasodilation\textsuperscript{235}. Insulin administered i.v. in humans increases total limb blood flow\textsuperscript{245}. Furthermore, impaired vasodilation is a characteristic of individuals with type 2 diabetes and increases the risk of atherosclerosis development. Insulin therapy in type 2 diabetic patients stimulates vasodilation and increases blood flow\textsuperscript{246}.

1.9.3 Insulin stimulates ET-1 and induces vasoconstriction.

On the other hand, insulin also induces vasoconstriction\textsuperscript{247}. Insulin stimulates the production and secretion of ET-1, a vasoconstrictor released from the vascular endothelium, in endothelial cells through increases in ET-1 gene expression\textsuperscript{248}. \textit{In vivo}, insulin infusion increased plasma ET-1 levels and induced hypertension in rats\textsuperscript{249}. It has also been reported that insulin stimulates vasoconstriction via ET-1 during NOS inhibition\textsuperscript{250} and that this response is mediated by MAPK\textsuperscript{251}. Furthermore, obese and insulin resistant individuals have excess circulating levels of ET-1\textsuperscript{252}. Taken together, these studies suggest that insulin resistance and the associated hyperinsulinemia may stimulate ET-1 production by endothelial cells. In the insulin resistant state the balance between NO and ET-1 production by insulin is altered in favour of ET-1 and results in vasoconstriction. Therefore, as insulin has opposing vasodilator and
vasoconstrictor actions, the net effect of insulin on blood pressure is minimal in healthy individuals.  

1.9.4 Insulin inhibits platelet function.

Human platelets contain insulin receptors, which are involved in the regulation of platelet function. Furthermore, eNOS is also present in human platelets and can be stimulated by insulin to increase NO production, which decreases platelet adhesion and aggregation. Studies have also shown that insulin inhibits platelet aggregation, perhaps in part through stimulation of NO production, in non-obese individuals, however this effect was not present in insulin resistant obese and hypertensive individuals. Therefore, an inhibitory effect on platelet aggregation may contribute to a protective effect of insulin on the vasculature.

1.9.5 Insulin has anti-inflammatory effects.

Initially, from epidemiological studies, insulin was thought to increase the risk of atherosclerotic CVD. It was suggested that insulin stimulation of the MAPK signalling pathway promoted inflammation and thrombosis by stimulating monocyte migration and vascular cell expression of PAI-1. However, the majority of studies have demonstrated that insulin has important anti-inflammatory properties (reviewed in ) and that it is insulin resistance that is detrimental.

Insulin administration in mice suppressed macrophage cholesterol synthesis and content, macrophage lipid peroxide content and ROS generation. Furthermore, in other animal studies insulin suppressed endotoxin- and thermal injury- induced pro-inflammatory transcription factors and genes. In endotoxin- treated human macrophage-1 cells, the stimulated increase in apoptosis, TNFα, and IL-1β secretion was decreased by insulin. Insulin decreased the expression of intracellular adhesion molecule-1 (ICAM-1) in human aortic endothelial cells through a NOS-dependent mechanism. Recent studies in humans have highlighted the protective effect of insulin in vivo on blood markers of inflammation and thrombosis. Insulin
infusion decreased plasma levels of ICAM-1\textsuperscript{262}, monocyte chemoattractant protein-1 (MCP-1)\textsuperscript{262}, tissue factor\textsuperscript{263}, plasminogen activator inhibitor-1 (PAI-1)\textsuperscript{262,263}, and C-reactive protein\textsuperscript{264}. Insulin infusion also inhibited NF\kappa B by stimulating I\kappa B in mononuclear cells\textsuperscript{262}. These changes could be seen in obese subjects (who are generally insulin resistant) and were not due to insulin-induced changes in glucose as normoglycemia was maintained.

1.9.6 Effect(s) of insulin on proliferation and migration.

Insulin-stimulated VSMC proliferation may be explained by activation of the MAPK signalling pathway as insulin and several other growth factors are known to activate this pathway in VSMCs\textsuperscript{265}. MAPKs phosphorylate other kinases, including regulatory transcription factors required for the expression of genes involved in proliferation. Insulin has a mitogenic effect in VSMCs\textsuperscript{266}. On the other hand, the majority of \textit{in vivo} studies report that insulin does not affect\textsuperscript{215,267} or even decreases\textsuperscript{268} VSMC proliferation. For example, hyperinsulinemia (10U/day), without changes in glycemia, in normal rats did not affect DNA synthesis two and four days after aortic injury\textsuperscript{215}, whereas in another study insulin (6U/day) treatment for four days after aortic injury decreased DNA synthesis in normal rats\textsuperscript{268}. The mechanism of this effect was not addressed but an effect of hypoglycemia was suggested as an explanation. However, in contrast, our laboratory has previously shown that insulin (5U/day) administration in normal rats increases VSMC proliferation after carotid balloon injury at seven days post-injury\textsuperscript{133}.

The effect of insulin on VSMC migration is controversial, as studies report that insulin either stimulates\textsuperscript{269,270} or inhibits\textsuperscript{271-273} VSMC migration \textit{in vitro}. However, the effect of insulin appears to be time- and dose-dependent; acute treatment at lower doses\textsuperscript{271,272} was inhibitory whereas chronic\textsuperscript{269} or supraphysiological\textsuperscript{270} doses were stimulatory. This may be explained by the fact that high levels of insulin stimulate IGF-1 receptors to mediate VSMC migration\textsuperscript{50}. Chronic insulin treatment can also induce insulin resistance\textsuperscript{274}, which may prevent insulin’s
inhibitory effect on VSMC migration. Insulin’s inhibitory effect on VSMC migration was abolished by NOS inhibitors\(^{272}\), suggesting a NO-dependent mechanism.

1.10 Resveratrol

1.10.1 General effects of RSV.

RSV (trans-3,5,4’-trihydroxystilbene) is a natural phytochemical found in several plant species, including grapes, and is subsequently present in high concentrations in red wine. Microbial infections, ultraviolet radiation, and chemical stressors induce RSV synthesis (reviewed in \(^{275}\)). Currently, RSV is commercially available as a dietary supplement. The presence of RSV in red wine lead to speculation that RSV may partly explain the “French Paradox”, which is the observation that mortality from cardiovascular disease is relatively low in France, despite high consumption of saturated fats, perhaps in relation to moderate red wine consumption. Numerous studies have been performed and have demonstrated that RSV, at least in vitro and in animal studies, has many properties that would support this hypothesis. Furthermore, from these studies, several beneficial health effects of RSV have been reported\(^{275}\), including anti-oxidant activity, cancer prevention, lifespan extension, and anti-diabetic and anti-cardiovascular disease properties. However, very few studies were performed in humans.

The cardiovascular benefits of RSV include protection against LDL oxidation, increasing high-density lipoprotein (HDL) cholesterol, inhibition of platelet aggregation and/or adhesion, and suppression of proliferation or hypertrophy of VSMCs and ECM synthesis (reviewed in \(^{275}\)), which will be discussed in the following sections. RSV has both estrogen-receptor agonist and antagonistic activity depending on the system, therefore its estrogen-like effect could also explain its cardio-protective ability as estrogen stimulates NO release\(^{276}\). RSV is more widely known for its anti-cancer properties. Studies have shown inhibition of cellular events associated with cancer initiation, promotion, and progression (reviewed in \(^{277}\)). RSV is recognized as an activator of AMPK \(^{278, 279}\), an energy sensor and suppressor of proliferation in many different cell
types. This suggests that RSV’s anti-cancer and anti-atherosclerotic activity (i.e. suppression of VSMC proliferation) may involve AMPK activation.

There are fewer studies examining the possible anti-diabetic effects of RSV, however interest has been rising in recent years. Oral RSV in Streptozotocin-induced diabetic rats resulted in a significant lowering of plasma glucose and lipid levels and stimulated glucose uptake in skeletal muscle, suggesting an insulin-like effect. In a very recent study, RSV increased insulin sensitivity and AMPK activity in mice fed a high-calorie diet. We have shown that RSV also stimulates glucose uptake in L6 skeletal muscle cells in a SIRT1- and AMPK-dependent manner.

Sirtuins are a family of histone/protein deacetylases shown to be involved in lifespan extension following caloric restriction in many organisms, from yeast to mammals. Many studies suggest that RSV is an activator of SIRT1 (reviewed in), however this concept has been challenged as very recent studies using new biochemical assays suggest that RSV is not a direct activator of SIRT1.

1.10.2 Effects of resveratrol on atherosclerosis.

Generally the effect of RSV on atherosclerosis is thought to be protective, however one early study reported that RSV (0.6mg/kg/day; gavage) promoted atherosclerosis in diet-induced hypercholesterolemic rabbits without changes in plasma lipoprotein-cholesterol (VLDL, LDL, HDL) concentrations. In contrast, another study in hypercholesterolemic rabbits demonstrated that RSV (3mg/kg/day) given in the drinking water suppresses atherosclerosis, however as in the previous study, plasma lipid levels were not affected. The difference between the studies may be explained by the different doses of RSV given or the frequency of RSV administration.

Subsequent reports in transgenic mouse models also showed that RSV has a protective effect against atherosclerosis. In Fukao et al., RSV (~1.5mg/kg/day; in food) decreased atherosclerotic plaque formation and laser-induced thrombus size in ApoE/LDLR knockout mice, without changes in plasma lipid (total cholesterol) levels. Atherogenic lesion formation
was also decreased in ApoE knockout mice given RSV (~32mg/kg/day; in food), however in this study total- and LDL-cholesterol levels were decreased while HDL- cholesterol levels were increased in the RSV group\textsuperscript{288}. Vascular cell adhesion molecule- 1 (VCAM- 1) and ICAM- 1 levels in the atherosclerotic vessel were also decreased with RSV.

Polyphenol mixtures containing low levels of RSV also inhibit atherosclerosis in ApoE knockout mice\textsuperscript{289, 290}. Plasma lipids were measured only in Norata et al\textsuperscript{289}, however no changes were found. From these studies it seems that RSV is vasculoprotective and that changes in plasma lipid levels are only observed with the highest doses of RSV.

1.10.3 Effects of resveratrol on restenosis.

RSV (4mg/kg/day; intragastric) was initially shown to decrease neointimal growth after arterial injury in rabbits\textsuperscript{291}. In the same study RSV decreased VSMC proliferation \textit{in vitro}, which was suggested as the mechanism to decrease neointimal formation. In a subsequent study Gu et al\textsuperscript{292} demonstrated that RSV (10mg/kg/day; gavage) reduced neointimal growth after aortic balloon injury in rats. RSV also accelerated re- endothelialization and increased eNOS mRNA expression in the aorta after injury; however, at a dose of 50mg/kg/day, RSV only decreased neointimal growth, indicating a dose- dependent effect. More recently, RSV (1mg/kg/day) was shown to reduce neointimal growth after carotid balloon injury in rats when administered in an emulsion system via i.p. injection\textsuperscript{293}. Furthermore, other AMPK activators (adiponectin\textsuperscript{294}, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR)\textsuperscript{295}), have been reported to inhibit restenosis after angioplasty in animals and humans.

1.11 Mechanism of Resveratrol Action in the Vasculature

1.11.1 General resveratrol signal transduction.

RSV’s molecular mechanism of action is not entirely clear. RSV is lipophilic and can easily cross the blood brain barrier and affect the central nervous system. RSV can enter the cell through simple diffusion\textsuperscript{296} and more recent studies have identified several receptors for RSV.
binding, including an aryl hydrocarbon receptor (AhR)\textsuperscript{297}, a sulfonylurea receptor (RSV binding blocks SUR1/K(IR)6.2 K(ATP) channels)\textsuperscript{298}, and the estrogen receptor in the vascular endothelium\textsuperscript{299}. As previously mentioned, RSV is most well known to act through intracellular targets such as SIRT1, AMPK, and MAPK (please see Figure 1.2 for a summary of RSV signalling). A major difference between insulin and RSV signalling is that PI3K regulates a large portion of insulin action whereas AMPK appears to be a key component of RSV action\textsuperscript{281}. A growing number of studies also support a role for RSV in stimulating NO production, which will be addressed in the following sections. A ‘classical’ RSV signal transduction pathway has not been defined as RSV affects countless signalling molecules with its effect varying with cell type. Therefore, I have limited the discussion to the most relevant signalling molecules in the vasculature as they pertain to each of the following sections.

1.11.2 Resveratrol stimulates NO production and induces vasodilation.

RSV exerts several cardiovascular protective effects by stimulating NO production. It has been reported that RSV enhances expression and activity of eNOS in endothelial cells\textsuperscript{300}, EPCs\textsuperscript{292}, and \textit{in vivo} in the heart\textsuperscript{301}. RSV decreased iNOS expression in macrophages\textsuperscript{302}, however it also enhanced cytokine-induced iNOS expression in rat VSMCs\textsuperscript{303}. RSV has been shown to increase NO production through an estrogen receptor\textsuperscript{304} and calcium\textsuperscript{305} dependent mechanism in endothelial cells. Furthermore, RSV activates AMPK\textsuperscript{282} and AMPK has been reported to activate eNOS through serine phosphorylation\textsuperscript{306}. For example, in endothelial cells, RSV induced eNOS activation and subsequent NO production through an AMPK-dependent mechanism\textsuperscript{142}. Another possible mediator of RSV-induced NO production is SIRT1 as RSV increased eNOS expression through a SIRT1-dependent manner in both cultured mouse arteries and human coronary arterial endothelial cells\textsuperscript{307}.

The vasorelaxant properties of RSV are, in part, mediated by NO. For example, chronic oral administration of RSV in rats induced significant vasodilation through a NO-dependent
 mechanism as L-N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) (a NOS inhibitor) co-
administration prevented the effect of RSV\textsuperscript{308}. RSV also induces vasorelaxation in the aorta of
hypertensive rats by decreasing superoxide production\textsuperscript{308} (superoxide inactivates NO). RSV has
also been shown to inhibit ET- 1- induced MAPK activity through upregulation of cGMP/ PKG
activity in VSMCs\textsuperscript{309}, down- regulate ET- 1 evoked protein tyrosine phosphorylation to inhibit
MAPK in porcine coronary arteries\textsuperscript{310}, and induce vasorelaxation\textsuperscript{311}. Similar to insulin, RSV
improves blood pressure in models of hypertension\textsuperscript{312}, however RSV treatment alone may not
have an effect on systolic blood pressure in normal rats.

1.11.3 Resveratrol inhibits platelet function and thrombus formation.

RSV is also an effective inhibitor of platelet adhesion and aggregation (reviewed in\textsuperscript{275}).
RSV inhibited platelet aggregation in human whole blood\textsuperscript{313}, in platelets isolated from human
plasma\textsuperscript{314}, and in experimentally induced hypercholesterolemic rabbits\textsuperscript{314}. RSV modulates
platelet activity through multiple mechanisms; 1) inhibition of platelet adhesion to collagen\textsuperscript{315}, 2)
inhibition of calcium influx into thrombin- stimulated platelets\textsuperscript{314}, and 3) reduction of tissue
factor (a cell surface glycoprotein that initiates blood coagulation) activity in endothelial cells
and monocytes\textsuperscript{316}. RSV treatment also decreased TNF\alpha- induced VCAM- 1 expression\textsuperscript{317}, which
could potentially reduce leukocyte adhesion to the vascular endothelium. Recently, RSV was
shown to activate eNOS in platelets isolated from human plasma to stimulate NO production and
inhibit platelet function\textsuperscript{318}, which could represent a source of NO after arterial injury.

The mechanism of the anti- platelet effect of RSV may involve cyclooxygenase (COX)
inhibition\textsuperscript{319}. The COX pathway promotes the formation of prostaglandins from arachidonic
acid, which initiate VSMC contraction of blood vessels and promote fever and pain as part of the
inflammatory response to injury. RSV is a competitive inhibitor of COX\textsuperscript{320} and prevents the
release of prostaglandins and thromboxanes\textsuperscript{321, 322}. Thromboxanes work with prostaglandins to
promote vasoconstriction and activate platelets in blood vessels to form a thrombus. RSV
inhibits thrombus formation by inhibiting thromboxane B2/ 12- hydroxyeicosatraenoate, which is specifically responsible for platelet aggregation induction and contraction of arteries\textsuperscript{322}. RSV also reduced laser-induced thrombosis in ApoE/ LDLR knockout mice\textsuperscript{287}.

1.11.4 Resveratrol has anti-oxidant effects.

ROS are essential in cell signalling and regulation at low concentrations, and affect gene transcription, protein synthesis, and cell function. However, an overabundance of ROS causes oxidative stress, which is one of the most important contributors to vascular damage and endothelial dysfunction. An excellent example of this oxidative stress is the production of superoxide and NO by endothelial cells, activated macrophages, and neutrophils during the inflammatory response. As previously discussed, superoxide and NO react to produce the oxidant peroxynitrite, which is involved in the development of atherosclerosis. Therefore, inhibition of vascular ROS can enhance the bioavailability of NO and protect against vascular disease.

LDL oxidation is a main cause of endothelial injury. RSV protects lipids from peroxidative degradation\textsuperscript{323} and prevents oxidized LDL uptake into the vascular wall\textsuperscript{324}. Protection of LDLs from peroxidation by RSV may be mediated through chelation of copper ions\textsuperscript{325} and by free radical scavenging\textsuperscript{326}. Ferrylmyoglobin and peroxynitrite also oxidize LDLs and RSV has been shown to protect against their oxidative effects\textsuperscript{327}. In addition, RSV induces intracellular anti-oxidant enzymes such as glutathione reductase and superoxide dismutase to decrease ROS levels\textsuperscript{328}.

1.11.5 Resveratrol has anti-inflammatory effects.

Numerous studies have demonstrated a significant anti-inflammatory effect of RSV. RSV’s anti-inflammatory properties are partly explained by its anti-oxidant properties, which were addressed in the previous section. RSV decreases the expression of pro-inflammatory markers (monocyte chemoattractant protein-1 (MCP-1), MIP-1, endothelin-1 (ET-1)) and
markers for macrophages and lymphocytes in the vascular wall. RSV also suppresses IL-6 gene expression and protein synthesis in macrophages. RSV decreases the expression of the adhesion molecules ICAM-1 and VCAM-1. More recently, in humans, RSV decreased NFκB, p47phox protein expression, and c-jun NH2-terminal kinase-1 (JNK-1) mRNA expression in mononuclear cells, demonstrating that RSV has a suppressive effect on inflammation as well as oxidative stress. Furthermore, the anti-inflammatory and anti-oxidant effects of RSV may both be explained, at least in part, by activation of AMPK. As an AMPK activator, RSV could decrease fat accumulation in insulin sensitive tissues, thereby quenching the source of inflammation.

1.11.6 Resveratrol inhibits proliferation and migration.

Several studies report that RSV inhibits the proliferation of VSMCs. Mainly, RSV decreases cell proliferation by inhibiting cyclin-dependent kinases (CDKs), (CDKs regulate cell cycle progression and DNA synthesis) and activating CDK inhibitors and tumour suppressor genes (p53 and p21). For example, RSV decreases VSMC proliferation by blocking the cell cycle at the G1/S transition and increasing p53 and p21 levels.

It appears that RSV utilizes different signalling pathways to inhibit proliferation, however it is important to remember that these pathways must converge upon common downstream regulators of the cell cycle, such as CDKs and CDK inhibitors. RSV’s inhibitory effect on VSMC proliferation has been reported to be mediated by the estrogen receptor and to be NO-dependent (associated increase in iNOS expression). Moreover, ET-1-evoked VSMC proliferation and MAPK phosphorylation were inhibited by RSV through a cGMP-dependent mechanism. MAPK induces VSMC proliferation and contraction in blood vessels and RSV has been shown to inhibit ERK1/2, JNK1, and p38 MAPK kinase phosphorylation and activity in porcine coronary arteries. Moreover, RSV has also been shown to inhibit cell proliferation through AMPK activation. Thus MAPK inhibition or AMPK activation may represent NO-
independent mechanisms to decrease cell proliferation. Cells that initiate apoptosis pathways do not undergo proliferation and thus RSV may affect proliferation indirectly, by stimulating apoptosis. Studies show that RSV induces or does not affect VSMC apoptosis, which may depend on the concentration of RSV.

In contrast to VSMCs, RSV stimulates proliferation in endothelial cells. Klinge et al reported that RSV activated MAPK and eNOS through an estrogen receptor-dependent mechanism in human umbilical vein endothelial cells. RSV also increased proliferation, migration, and eNOS expression in EPCs. However, at very high concentrations, RSV induced apoptosis in endothelial cells.

RSV also inhibits VSMC migration while promoting endothelial and EPC migration. The expression of MMPs, as previously mentioned, are essential for VSMC migration and contribute to the intimal thickening that is characteristic of restenosis. MMP expression is under MAPK and NFκB control. RSV inhibits MMP-2 and MMP-9 expression in human glioblastoma and vascular smooth muscle cells respectively. More specifically, in human VSMCs treated with TNFα, RSV decreased MMP-9 expression through a reduction in the binding of the transcription factors NFκB and AP-1, suggesting that RSV may decrease VSMC migration by NFκB-mediated inhibition of MMPs. As discussed earlier, RSV activates AMPK and there is some evidence that AMPK activation decreases cell migration, in addition to proliferation. Furthermore, maintenance of the differentiated state of VSMCs prevents their transition to a migratory phenotype and results in decreased VSMC migration. As mentioned previously, inhibition of the mTOR/ S6K1 pathway by rapamycin (mTOR is downstream of AMPK) has been shown to promote VSMC differentiation. RSV is also an inhibitor of mTOR signalling, thus representing yet another mechanism by which RSV may decrease VSMC migration.
1.12 Summary

Type 2 diabetes and obesity-associated metabolic syndrome, which are characterized by insulin resistance, are potent risk factors for atherosclerosis. Both conditions are also risk factors for restenosis after revascularization procedures used for treatment of atherosclerosis. The overall effect of insulin on the vasculature has been a point of controversy, however the prevailing effect of insulin on atherogenesis in vivo is now considered to be protective (PI3K mediated) and instead, insulin resistance is considered to be harmful (MAPK mediated) because of the unresponsiveness to insulin action, including its vascular anti-inflammatory properties. Previous studies from our laboratory support a protective role of insulin against restenosis. Furthermore, studies from other authors support that RSV may also protect against restenosis and atherosclerosis. The mechanisms underlying the protective effects of insulin or RSV are not fully understood. NO is a critical regulator of vascular homeostasis and its production has been reported to be stimulated by both insulin and RSV in numerous studies, thus representing a likely target of insulin or RSV action.

1.13 Rationale and Significance of the Studies

Atherosclerotic cardiovascular disease is a leading cause of morbidity and mortality worldwide\cite{1} and revascularization procedures, such as angioplasty and stenting, often result in restenosis. Type 2 diabetes has also become a global epidemic\cite{348} and macrovascular complications are the major cause of mortality in diabetic patients\cite{349}. Type 2 diabetes and the related metabolic syndrome, a cluster of risk factors for both atherosclerosis and type 2 diabetes, are both characterized by insulin resistance, and it is now well established that insulin resistance itself is a potent risk factor for atherosclerosis and restenosis after percutaneous interventions\cite{124}. Although drug-eluting stents have reduced the rate of restenosis in non-diabetic individuals, results are still worse in persons with type 2 diabetes\cite{10}.
In vitro, insulin has mitogenic effects on VSMCs\textsuperscript{265}, however previous studies in our laboratory\textsuperscript{133, 134}, as well as by others\textsuperscript{222}, have shown that insulin decreases neointimal growth after rat carotid balloon injury, a model of restenosis, thereby demonstrating a protective effect of insulin on the vasculature. NO production is important for vascular function and has been shown to diminish neointimal growth after injury\textsuperscript{105, 149}. Furthermore, insulin has protective effects on the endothelium by stimulating eNOS and NO production\textsuperscript{235, 236}. NOS inhibition has also been reported to abolish the effect of insulin to inhibit VSMC migration \textit{in vitro}\textsuperscript{272}. However, whether the mechanism of action of insulin to limit neointimal growth involves NO has not been elucidated.

Another promising compound to prevent restenosis is RSV, an AMPK activator with both anti- CVD and anti-diabetic properties. RSV also decreases neointimal formation after arterial injury\textsuperscript{291-293}, similar to insulin\textsuperscript{133, 134}, and has been shown to enhance the expression of eNOS in injured aortas\textsuperscript{292}, however NO was not demonstrated to be causally linked to the inhibition of neointimal growth. Moreover, these studies did not focus on investigating the mechanism or signalling molecules involved in RSV action \textit{in vivo}. In addition, reports suggest that RSV action is Akt- independent, suggesting that RSV would remain effective in a state of insulin resistance, thus providing an advantage over insulin. Therefore, it is important that the mechanism of action of RSV to decrease neointimal formation is addressed.

The three studies in this thesis were performed to specifically address the mechanism of action of insulin or RSV to protect against restenosis, and whether NO production mediates these effects using the NOS inhibitor L-NAME. To do this, we utilized an \textit{in vivo} experimental model of restenosis, carotid balloon angioplasty, in normal rats in all three studies. Although the rat model has minimal inflammatory and thrombotic components when compared with human restenosis\textsuperscript{21}, and does not allow the study of long- term vascular remodelling, it is the best characterized model of arterial injury and restenosis\textsuperscript{17} and facilitates the investigation of the
mechanism of insulin’s and RSV’s effect on restenosis. These studies may provide initial evidence for the clinical potential of insulin or RSV or their downstream mechanisms in the prevention of restenosis after angioplasty.

1.14 General Hypothesis

Insulin and RSV have protective effects against restenosis after arterial injury and their protective effects are mediated by NO.

1.15 Specific Aims

This thesis consists of three studies that examined the protective effects of insulin or RSV in a model of restenosis in rats, and the role of NO as a possible mediator of these effects.

**Study 1.** To investigate the effects of insulin treatment on neointimal growth after arterial injury, independent of glucose- lowering and oral glucose or sucrose.

**Study 2.** To examine the consequences of NOS inhibition, with the *in vivo* inhibitor L-NAME, on the effect of insulin to inhibit neointimal formation.

**Study 3.** To determine the effect of RSV on the processes involved in neointimal formation after arterial injury, and to investigate the consequences of NOS inhibition on these effects of RSV.
Figure 1.1. Signal transduction by the insulin receptor.
Insulin binding to its receptor results in tyrosine phosphorylation (-Tyr-PO32) of the insulin receptor, IRS (Insulin Receptor Substrates)1-4 and Shc (Src homology collagen-like). Phosphatidylinositol-3 Kinase (PI3K) and GRB bind to phosphorylated IRS, which activate the PI3K and MAPK (Mitogen Activated Protein Kinase) pathways. GRB also binds to phosphorylated Shc. Akt is downstream of PI3K.
Figure 1.2. Proposed signal transduction by resveratrol.
Resveratrol action in the vasculature may involve binding to the estrogen receptor to stimulate nitric oxide (NO) production\textsuperscript{304}. The increase in NO production is possibly mediated by activation of SIRT1\textsuperscript{307} and/or 5'- AMP- Activated Protein Kinase (AMPK)\textsuperscript{142}. Furthermore, activation of AMPK may lead to inhibition of Mitogen Activated Protein Kinase (MAPK) pathway\textsuperscript{295}, however there are also reports that resveratrol can stimulate MAPK to improve endothelial function through an estrogen receptor- dependent mechanism\textsuperscript{339}.
2

General Methods

2.1 Animals

For all studies, normal male Sprague-Dawley rats (Charles River, Quebec, Canada) weighing 350-425g were used for experiments. Male rats were studied since estrogen has been shown to attenuate intima thickening following carotid injury in rats and consequently the vessel response to injury is greater in males\(^{350}\). The rats were housed in the University of Toronto’s Department of Comparative Medicine. They were exposed to a 12h light/dark cycle. The rats used in Study 1 were fed a normal rodent diet (Dyets Inc. 100741; 18% Protein diet with 70% carbohydrate and 12% fat; powder form; Dyets Inc., Bethlehem, PA) to allow comparison with the study by Kim et al\(^{133}\). The rats used in Studies 2 and 3 were also fed a normal rodent diet that differed slightly from Study 1 (Teklad Global 2018; 21% Protein Diet with 64% carbohydrate and 14% fat; pellet form; Harland Teklad Global Diets, Madison, WI). The animal care committee of the University of Toronto approved all procedures.

2.2 Surgical Procedures

2.2.1 Subcutaneous Implant Procedure.

For Studies 1 and 2 rats were implanted with either insulin-releasing implants (5U/day for Study 1 and 3U/day for Study 2; 10-14% bovine insulin, 86-90% palmitic acid; 26 ± 2mg/capsule, LinShin Canada Inc.) or blank implants (100% palmitic acid; 26 ± 2mg/capsule) two days (Study 1) or three days (Studies 2 and 3) before balloon angioplasty using isoflurane gas.
The implants were placed at the back of the rat’s neck using a hypodermic needle. Rats receiving insulin capsules were also given subcutaneous (s.c.) 0.25ml glucagon and 2.5ml 50% glucose solution i.p. to prevent hypoglycemia caused by tiny pieces that broke away from the capsule during implantation. Previous reports have demonstrated sustained release and action of the insulin implants (>40 days) after implantation\textsuperscript{351}. The blood glucose of the rats was monitored frequently (every 2 hours) throughout the day using a glucometer (Glucometer Elite, Bayer Inc.). The insulin- treated rats were also provided with 40% glucose in their drinking water to maintain normoglycemia. Analgesic (Buprenorphine) was given at the end of each surgical procedure.

2.2.2 Intraperitoneal Catheterization for Glucose Infusion.

In Study 1, where the rats received high- dose insulin (5U/day), an incision was made on the dorsal surface of the neck and also through the abdominal muscles on the mid- lateral side of the rat abdomen after the carotid balloon injury procedure. Sterile tubing (polyethylene- 50 (PE-50); Cay Adams, Boston, MA) extended with a segment of silastic tubing (length of 2cm, internal diameter of 0.02inches; Dow Corning, Midland, MI) was tunneled subcutaneously from the neck incision, along the side, and through the abdominal muscles into the peritoneal cavity of the rat. The tubing was secured with a suture in the abdominal muscle wall and subcutaneous tissue, and exteriorized at the back of the neck. Saline was flushed through the line to ensure proper infusion and the incisions were closed. The insulin- treated rats received variable infusions of 25% glucose solution (50% dextrose diluted in saline (0.9% sodium chloride) to maintain euglycemia, where the infusion rate was adjusted daily based on blood glucose levels. Control rats received a saline infusion (0.9% sodium chloride).

2.2.3 Osmotic Pump Implant Procedure.

In Studies 2 and 3 rats were implanted with osmotic pumps for RSV (4mg/kg/day dissolved in polyethylene glycol- 300 and dimethyl sulfoxide (9:1)) or L-NAME (2mg/kg/day or 10mg/kg/day dissolved in saline) treatments three days before balloon angioplasty using
isoflurane gas. Osmotic pumps allow continuous delivery of RSV or L-NAME. An incision was made on the dorsal side of the rat, at a different site from the capsule implant, and a pocket was made s.c. with a blunt dissector to insert the osmotic pump, and the wound was closed.

2.2.4 Carotid Balloon Injury.

Arterial injury was performed by balloon angioplasty only on rats with blood glucose levels greater than 4.0mM. Injury to the left common carotid artery (LCCA) of the rat was induced by means of an inflated balloon catheter. Rats were anesthetized with a ketamine: xylazine: acepromazine cocktail (87:1.7:0.4mg/ml, 1ul/g body weight) (Study 1) or isoflurane (Studies 2 and 3). A midline incision of the neck was made to expose the left external carotid artery and rats underwent balloon injury where a 2F Fogarty balloon catheter (Baxter) was introduced into the LCCA and then inflated (30µl of saline) and withdrawn while rotating to denude the vessel of endothelium. This was performed four times. Then the LCAA was ligated and the wound was closed.

2.2.5 Aortic Balloon Injury.

Injury to the aorta of the rat was induced by means of an inflated balloon catheter. In brief, after undergoing isoflurane anesthesia, rats underwent balloon injury of the thoracic aorta. A 2F Fogarty balloon catheter (Baxter) was introduced via the LCCA into the aorta and then inflated and withdrawn three times. Then the LCAA was ligated and the wound was closed.

2.2.6 Vessel Cannulation.

Three days before the hyperinsulinemic-euglycemic clamp, rats were anesthetized with a ketamine: xylazine: acepromazine cocktail (87:1.7:0.4mg/ml, 1ul/g body weight) (Study 1) or isoflurane (Study 3). Indwelling catheters were inserted into the right internal jugular vein for infusions and the LCCA for sampling. Polyethylene catheters (PE-50; Cay Adams, Boston, MA), each extended with a segment of silastic tubing (length of 3cm, internal diameter of 0.02inches; Dow Corning, Midland, MI), were used. The venous catheter was extended to the level of the
right atrium and the arterial catheter was advanced to the level of the aortic arch. Both catheters were tunneled subcutaneously and exteriorized. The catheters were filled with a mixture of saline and heparin (1000 U/ml) to maintain patency and were closed at the end with a metal pin. The rats recovered for three days before experiments.

2.3 Blood and Vessel Sample Collection

After arterial injury rats were sacrificed at the appropriate time-point in either the fed or fasted state in order to obtain blood samples in different metabolic states. Blood glucose was monitored throughout the treatment using a glucometer and measured at the same time of the day (~10a.m.). Random fed glucose levels were also measured to ensure that normoglycemia was maintained throughout the fed state. These values were converted to plasma glucose assuming a normal hematocrit. To obtain fasting blood glucose levels, rats were fasted once for ~16 h (tap water/40% glucose solution/10% sucrose solution was given without food). Under general anesthesia, blood samples were collected via cardiac puncture for insulin, triglyceride (TG), and FFA measurements. Following blood sample collections, rats were sacrificed via anesthetic overdose.

2.3.1 Fixed Vessel Collection.

To obtain fixed vessels for morphometric analysis and staining, rats were subjected to retrograde perfusion through the abdominal aorta with Krebs- Ringer solution and then with 10% buffered formalin at the physiologic pressure (110-120mmHg). A 2cm length vessel was excised from the middle of each of the left and right common carotid arteries to ensure that the vessel cross-sections used for analysis had the most even vessel damage and were within the arterial region of the vessel which never completely re-endothelializes. Immediately following removal, the vessel was immersed in 10% buffered formalin for 24 hours and then transferred to phosphate buffered saline (PBS). All vessels were embedded in paraffin and divided into two blocks by cutting the midsection of the artery. Cross-sections were taken from the midsection.
2.3.2 Frozen Vessel Collection.

To obtain fresh vessel samples for Western blot, a 2cm length vessel was excised from the middle of each of the left and right common carotid arteries, rinsed in saline to remove excess blood and immediately frozen in liquid nitrogen and stored at -80°C.

2.4 Morphometric Measurements and Calculations

For all analyses in this thesis, a blinded coding system was used to avoid experimenter bias. Intimal and medial cross-sectional areas were measured on hematoxylin and eosin stained slides (prepared in the Histology/Pathology Core laboratory at The Centre for Modeling Human Disease (Toronto Centre for Phenogenomics, Toronto, ON, Canada)) and analyzed using a computer-assisted morphometric system (Simple PCI, Compix Inc.). Images of the vessels were obtained using a Nikon E600 microscope (Nikon, Mississauga, ON, Canada), digitized using a camera (model C4742-95-12N RB; Hamamatsu, Inc.). Two separate cross-sections were measured on each carotid artery and averaged. This average value was used to represent each individual rat.

Intimal area was measured as the area encompassed by the internal elastic lamina minus the lumen area (in this case lumen area was determined by tracing around the inside edge of the vessel and calculating the area inside). Medial area was measured as the area encompassed by the external elastic lamina minus the area encompassed by the internal elastic lamina (including lumen area). Lumen area was calculated by subtracting the intimal area and the medial area from the total area encompassed by the external elastic lamina (in this case total area was calculated using the external elastic lamina perimeter). This method of obtaining lumen area avoids artifacts due to paraffin embedding.
2.5 Histomorphometry/Matrix Staining

2.5.1 Cell Migration Assay.

Cell migration into the intima was measured four days after injury and intimal cells on the lumen surface were immunostained with an antibody against histone H1 (MAB1276; Chemicon) which does not permeate the internal elastic lamina and as a result stains only intimal cell nuclei\textsuperscript{45, 82}. This migration assay takes advantage of the fact that the first cells appear in the intima three to four days after injury\textsuperscript{82} and do not complete a round of replication until 24 hours later. An approximately 2cm centre section of the LCCA was cut lengthwise and pinned open on a Teflon card with the lumen side up. The arteries were rinsed with PBS and bathed in a solution of 0.3% hydrogen peroxide and cold methanol for 30 minutes to block endogenous peroxidase. Non-specific protein binding was blocked with 2% normal horse serum (diluted with 1% PBS: bovine serum albumin (BSA)) for 30 minutes. The arteries were then incubated with the primary antibody histone H1 monoclonal antibody (MAB1276, mouse anti-human nuclei and chromosomes, Chemicon International Inc) (diluted 1:100 in 1% PBS/BSA) for 30 minutes. This was followed by incubation with the secondary antibody, 1% biotinylated horse anti-mouse IgG (diluted in 1% PBS/BSA), for 30 minutes. Peroxidase labeling was carried out using an avidin-biotin complex (ABC Elite, Vector Laboratories) during a 30 minute incubation. Arteries were incubated in Tris (6.1g/L of distilled water, pH 7.6) for 5 minutes and then stained using 0.05% 3,3’-diabminobenzidine (DAB) in 0.05mmol/L Tris-HCl (pH 7.6) with 50µl of 30% hydrogen peroxide for 5 minutes. All steps were followed by PBS rinses in triplicate. At the end of the assay the arteries were mounted on glass slides and coverslips were applied with a 50:50 mixture of PBS: glycerol. The artery was visualized with light microscopy and the entire luminal surface of the vessel was counted with a minimum of 12 fields taken at a magnification of 200X. Each nucleus that was oriented parallel to the long axis of the vessel was counted, thus discounting medial cells which are perpendicular.
2.5.2 BrdU- Labelling.

To label proliferating neointimal cells, rats were injected i.p. with three doses of 25 mg/kg 5-bromo-2’-deoxyuridine (BrdU; Sigma) at 17, 9, and 1 hour before sacrifice. The LCCAs embedded in paraffin were sliced into 4µm thick sections and mounted on slides in the Histology/Pathology Core laboratory at The Centre for Modeling Human Disease (Toronto Centre for Phenogenomics, Toronto, ON, Canada). The vessel cross-sections were deparaffinized in xylene washes and rehydrated in 100% ethanol. To block endogenous peroxidase activity, the slides were immersed in a solution of 3% hydrogen peroxide dissolved in cold methanol. After repeated washes in distilled water, proteinase K (DAKO Corporation) was added to the cross-sections to digest proteins and expose DNA. Again the slides were rinsed in distilled water and immersed in 4N hydrochloric acid to denature DNA so that the antibodies could bind to the BrdU incorporated into the DNA. The slides were then rinsed in distilled water, followed by PBS for 10 minutes, and then incubated in the primary antibody (rat anti-BrdU antibody diluted in 1% normal horse serum and PBS solution, 1:40 dilution; DAKO Corporation) at 37°C in a humidified chamber for 60 minutes to stain BrdU incorporated into the DNA of proliferating intimal VSMCs. Following the incubation, the slides were washed with PBS and incubated with the secondary antibody (biotinylated anti-MoIgG HAM antibody diluted in PBS, 1:40 dilution; Vector Laboratories) at 37°C in a humidified chamber for 30 minutes. The slides were rinsed with PBS and incubated in the tertiary antibody (ABC Elite diluted in PBS, 1 drop/2.5ml PBS; Vector Laboratories) at 37°C in a humidified chamber for 30 minutes. Subsequently, the slides were washed with PBS and then a Tris solution (50mM pH 7.6 in distilled water) for 5 minutes, then immersed in DAB (Sigma) solution for 10 minutes. Immediately following, the reaction was stopped by immersing the slides in 70% ethanol for 5 minutes. The slides were again washed with PBS and counterstained with hematoxylin (hematoxylin diluted 1: 2 in distilled water; DAKO Corporation) solution for 3 minutes. The slides were dipped in acid
alcohol (10ml distilled water in 190ml 95% ethanol plus 8 drops of glacial acetic acid) followed by development with Scott’s Tap Water for 5 minutes. The slides were rinsed with PBS and dehydrated with successive washes of increasing concentrations of ethanol. Xylene was applied to the slides for 5 minutes (2 times) and then slides were mounted and coverslipped with Permount. The total number of BrdU- positive stained cells was counted in the intima of the vessel cross- sections under 400X magnification.

2.5.3 Terminal dUTP nick- end labeling (TUNEL) Assay.

The rate of cell apoptosis in the intima was measured using terminal dUTP nick- end labeling (TUNEL) on cross- sections. The staining was prepared in the Histology/ Pathology Core laboratory at The Centre for Modeling Human Disease (Toronto Centre for Phenogenomics, Toronto, ON, Canada). The vessel cross- sections were deparaffinized in xylene and rehydrated with decreasing concentrations of ethanol, followed by washes with distilled water and PBS. Proteinase K (10µg/ml in PBS; DAKO Corporation) was added to digest proteins and expose DNA. The vessels were then washed with distilled water and PBS, followed by incubation in a solution of 3% hydrogen peroxide dissolved in methanol for 30 minutes to block endogenous peroxidase activity. The slides were then washed with PBS, pre- incubated with 1X One- Phor- All Buffer (10X stock diluted to 1X with 0.1% TritonX- 100 in distilled water; Pharmacia) for 10 minutes, and then incubated in TdT solution (10µl 10X One- Phor- All Buffer + 6µl dilute biotin- 16- d UTP + 1µl 100 µ M d ATP + 1µl TdT enzyme + 82µl 0.1% TritonX- 100 in distilled water) in a humidified chamber for 2 hours at 37°C. The slides were washed with PBS and incubated in the ABC Complex solution (Vectastain) for 90 minutes at 37°C, followed by a wash with a solution of 0.1% TritonX- 100 in PBS. The slides were immersed in DAB (Sigma) solution for 15 minutes to develop the colour and counterstained with hematoxylin (hematoxylin diluted 1:2 in distilled water; DAKO Corporation) for 20 seconds, then washed with water and dipped in acid alcohol (10ml distilled water in 190ml 95% ethanol
plus 8 drops of glacial acetic acid) followed by development with Scott’s Tap Water for 1 minute. The slides were rinsed with PBS and dehydrated with successive washes of increasing concentrations of ethanol. Xylene was applied for 1 minute (3 times) and then slides were mounted and coverslipped with Permound. The total number of TUNEL- positive stained cells was counted in the intima of the vessel cross- sections under 400X magnification.

2.5.4 Extracellular Matrix Staining with Movat’s Pentachrome and Picrosirius Red.

Cross- sections taken 14 and 28 days after injury were also stained with Movat’s pentachrome or picrosirius red (PSR) dye for elastin and collagen respectively. PSR stains collagen fibers red and Movat’s pentachrome stains elastin fibers deep purple. The Movat’s Pentachrome and PSR staining was prepared in the Histology/ Pathology Core laboratory at The Centre for Modeling Human Disease (Toronto Centre for Phenogenomics, Toronto, ON, Canada). For the Movat’s Pentachrome stain, the vessel cross- sections were deparaffinized in xylene washes and rehydrated in 95% ethanol. The slides were immersed in Bouin’s solution for 1 hour at 60ºC, washed well under running water, rinsed in 0.5% acetic acid, and then stained with alcian blue for 5 minutes, followed by an additional rinse in 0.5% acetic acid. The slides were then washed in water and stained with Working Elastin stain for 8 minutes. The slides were incubated in 3% sod thiosulfate for 1 minute and then washed in distilled water. Biebrich Scarlet-Acid Fuchsin was used to stain the slides for 90 seconds followed by another wash under running water. 0.5% acetic acid was used to rinse the slides with subsequent differentiation in phosphotungstic acid for 5 minutes and an addition rinse with 0.5% acetic acid and then absolute ethanol. The slides were incubated in saffron stain for 5 minutes and followed by successive washes of absolute ethanol. Xylene was applied to the slides for 5 minutes (3 times) and then slides were mounted and coverslipped with Permound.
The protocol for PSR staining was similar to Movat’s Pentachrome with the following changes; after the deparaffinization and rehydration steps the slides were stained in Celestine blue for 5 minutes and then washed in water and stained in Harris’ haematoxylin for 5 minutes. The slides were again rinsed with water and dipped in acid alcohol (5 dips). Scott’s Tap Water was used to develop the slides for 1 minute. The slides were washed under running water and stained with PSR for 30 minutes and blotted dry, followed by subsequent dehydration, xylene application, and mounting.

To quantify the degree of collagen or elastin stain in the cross-sections of arteries, the colour/ intensity of the positively stained areas in the media and intima of the vessel was analyzed using a computer laboratory imaging system (NIS Elements BR 3.0, Nikon). A selected intensity of the stain was set as positive staining and used as a threshold. The software uses this threshold to identify the area of the vessel stained positively within a defined total area. The amount of collagen or elastin positively stained area was then expressed as a percentage of total area measured.

2.5.5 Evan’s Blue Staining.

To measure the re-endothelialized area at 14 days after aortic balloon injury, Evan’s Blue dye solution was introduced into the rat circulation via the abdominal aorta. Evan’s Blue binds to negatively charged albumin and circulates throughout the vasculature. While in circulation, this dye penetrates any vessel without an intact endothelial cell layer and stains that area blue, leaving the intact endothelium unstained (remains white). 1.0ml of 2.5% Evans Blue solution (diluted in distilled water) was injected with a syringe into rats via the abdominal aorta. Ten minutes after the injection, rats were sacrificed via anesthetic overdose and the aorta was perfusion-fixed for 15 minutes with 4% buffered formalin at physiologic pressure (110-120mmHg). To observe Evans Blue uptake macroscopically, the thoracic aorta was dissected.
out, opened longitudinally, and an image was captured with a digital camera. The re-endothelialized area was defined as the area not stained with Evans Blue dye. To quantify the area stained with Evans Blue on the longitudinal sections of the aorta, the colour/ intensity of the stained area on the luminal surface of the vessel was analyzed using a computer laboratory imaging system (NIS Elements BR 3.0, Nikon). A selected intensity of the stain was set as positive staining and used as a threshold. The software uses this threshold to identify the area of the vessel stained positively within a defined total area. The amount of positively stained area was then expressed as a percentage of total area measured. Cross- sectional areas were measured on hematoxylin and eosin stained slides from unopened sections of these aortas as described in the Morphometric Measurements and Calculations section 2.4.

2.6 Vessel Homogenization

The frozen vessels were pulverized using a mortar and pestle in liquid nitrogen. The powdered vessel was placed into 150µl of cell lysis buffer (1% sodium dodecyl sulfate (SDS), 1mM phenylmethysulfonyl fluoride, and 10 mg/ml leupeptin in 50 mM Tris buffer, pH 7.6). The sample was centrifuged (13000 rpm, 10 minutes, RT) and the supernatant was aliquoted for either protein assay or Western blot. The sample for Western blot was diluted 1:1 with electrophoresis sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris HCl).

2.7 Protein Assay

The Biorad Detergent- Compatible Protein Assay (Biorad, ON, Canada) was used to measure the protein concentration of each sample with BSA used as a standard. The assay is a colorimetric assay for protein concentration following detergent solubilization. The aliquoted samples for protein assay were thawed, vortexed, and kept on ice. Then the samples were diluted (1:3) with distilled water (in this case only 5µl of sample was needed) in a glass tube. Stock
BSA (1 mg/ml) was diluted accordingly with cell lysis buffer to prepare a standard curve ranging from 0 to 1 mg/ml. Then 20 µl of these BSA standard samples were transferred to glass tubes. 100 µl of the Reagent A solution (20 µl of Reagent S/ 1 ml of Reagent A) was added to each test tube and then vortexed, followed by the addition of 800 µl of Reagent B and vortexing. After 15 minutes samples were transferred to cuvettes and absorbance was read at 750 nm using a spectrophotometer.

2.8 Western Blot Analysis

Carotids were removed seven days after injury, homogenized, and processed as previously described in section 2.6. Protein samples of the appropriate concentration (25µg was used for all antibodies except total eNOS for which 60µg was used) were thawed, vortexed, and then subjected to sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis (10% polyacrylamide gel) for 90 minutes. Following electrophoresis separation, protein was transferred to polyvinylidene difluoride (PVDF) membranes in transfer buffer on ice for 90 minutes. The membranes were then blocked for 1 hour at room temperature in Tris-buffered saline- Tween (TBST) containing 5% non-fat dried milk, pH 7.4, followed by overnight incubation at 4°C with the appropriate primary antibody diluted in the blocking solution (anti-SM-MHC (Santa Cruz Biotechnology sc-6956 (1:1000)), anti- SM22α (Santa Cruz Biotechnology sc-18513 (1:500)), anti- SMC alpha- actin (Sigma A2547 (1:1000)), anti- total eNOS antibody (Santa Cruz Biotechnology sc-654 (1:500)), and anti- alpha tubulin (Cell Signalling Technology #2144 (1:1000)) antibodies, all specific for rat). After three washes with TBST (20 minutes each) to remove excess primary antibody, membranes were incubated with the secondary antibody horseradish peroxidase- conjugated goat anti- rabbit IgG (Santa Cruz sc-2004 (1:2000)) for 1 hour at room temperature. The membranes were then washed three times (5 minutes each) with distilled water and developed using enhanced chemiluminescence (Thermo
Scientific) and exposed to x-ray film. The bands obtained from immunoblotting were quantified by densitometry using Image J (National Institute of Health Research).

2.9 Flow Cytometry

Rat blood was obtained at three and seven days after carotid injury in separate rats by cardiac puncture. Flow cytometry was performed as in Fadini et al\textsuperscript{115} by our collaborator Dr. M.R. Ward in Dr. D.J. Stewart’s laboratory at St. Michael’s Hospital (Toronto, ON). Blood was drawn into Sodium Citrate (9:1 v/v) Vacutainer tubes (BD Biosciences). For each staining sample, 200 µl of blood was mixed with 1.0 mL RBC lysis buffer (eBioscience) and incubated for 10 min at room temperature in the dark. Tubes were centrifuged and the supernatant discarded. The remaining cells (pellet) were suspended in 100 µl of PBE staining buffer, and appropriate primary antibody (mouse anti-rat VEGFR2 (Novus Biologicals), rabbit anti-rat c-kit (Neuromics), and rabbit anti-rat sca-1 (Novus Biologicals) was incubated with the cells at room temperature in the dark for 30 minutes. Following washing (2x 1.0 ml PBE buffer) and centrifugation, cells were exposed to secondary antibody (FITC donkey anti-rabbit IgG) or Straptavidin-APC for 30 minutes in the dark. Following two additional wash and centrifugations steps, cells were suspended in PBS. The number of gated (defined) cells positive for the three markers was quantified by flow cytometry (Beckman Coulter Cytomics FC500) using FL1 (FITC for c-kit and sca-1) and FL4 (for VEGFR2) filters\textsuperscript{115}.

2.10 Hyperinsulinemic- Euglycemic Clamp

The rats were fasted overnight (~5pm to 9am). At -20 minutes and immediately before the infusion (Basal Period), blood samples were taken for measurements of plasma glucose and insulin. At the onset of the clamp, insulin (Humulin R, 5mU/kg.min) and glucose (variable rate) were infused through the jugular vein for two hours to maintain euglycemia while blood samples were taken from the LCAA for plasma glucose measurements. The total volume of blood
withdrawn was ~4 ml. After plasma separation, red blood cells were diluted at 1:1 in heparinized saline (4U/ml) and re-infused into the rats. At the end of the experiments, the rats were anesthetized with intravenous administration of ketamine: xylazine: acepromazine cocktail (87:1.7:0.4 mg/ml, 1ul/g body weight).

2.11 Blood Pressure Analysis

Systolic blood pressure was measured indirectly from the tail via the cuff technique, using the Visitech BP-2000 Blood Pressure Analysis System. This system determines the cuff pressure at which changes in blood flow occur during occlusion or release of the cuff. Each rat was placed in a restrainer on the heated platform (37°C) with the blood pressure cuff placed around the base of the tail. The restrainer was covered with a towel to avoid distraction for the rat. This was followed by a ‘rest period’ of approximately 15 minutes to lessen the effects of stress. Then 20 preliminary systolic blood pressure measurements were taken but not recorded to allow the rat to become accustomed to the increasing pressure of the tail cuff. Following the preliminary measurements, 10 systolic blood pressure measurements were recorded and the average was taken as the final value. Systolic blood pressure was measured before treatment and throughout at 4, 14, and 28 days after arterial injury.

2.12 Metabolic Parameters

2.12.1 Plasma Insulin.

Plasma insulin levels were determined by radioimmunoassay (RIA) using kits specific for rat insulin (Linco Research Inc, St. Charles, MO). The rat insulin RIA is a double antibody radioimmunoassay. Insulin in the sample competes with a fixed amount of ¹²⁵I-labeled insulin for binding sites on the specific antibodies. Bound and free insulin were separated by the addition of a second antibody immunoabsorbent followed by centrifugation and aspiration of the supernatant. The radioactivity of the pellet was then measured and was inversely proportional to the amount of insulin in the sample. A standard curve was first constructed using insulin
standards with known concentrations (0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 ng/ml) in duplicate. After samples were pipetted into appropriate tubes, $^{125}\text{I}$-insulin was added (100 µl to all tubes) followed by the rat insulin antibody (100 µl to all tubes). The tubes were then vortexed to ensure mixing and were incubated overnight (18-24 hrs) at 4°C. On the next day, 1.0 ml of precipitating reagent was added to all tubes followed by vortexing and incubating for 20 minutes at 4°C. The tubes were then centrifuged at 2500 rpm for 40 minutes. The supernatant was aspirated and the radioactivity in the pellet was counted for 4 minutes in a gamma counter (Beckman Instruments, Fullerton, CA, USA). The counts (B) for each of the standards and unknown were expressed as a percentage of the mean counts of the “0- standard” (Bo).

$$\% \text{ Activity Bound} = \frac{B \text{ (Standard or sample)}}{B_0} \times 100\%$$

The %B/Bo for each standard was plotted on the y- axis and the known concentration of the standard on the x- axis on log-log graph paper and a standard curve was constructed. The concentration of the unknown samples was determined by interpolation of the standard curve. The coefficient of interassay variation determined on reference plasma was less than 10%.

2.12.2 Plasma Free Fatty Acid.

Plasma levels of FFA were analyzed using a colorimetric kit under enzymatic reaction (Wako Industrials, Neuss, Germany). The method relies upon the acylation of coenzyme A by the fatty acids in the presence of added acyl- CoA synthetases (ACS). The acyl- CoA produced is oxidized by adding acyl- CoA oxidase (ACOD) which generates hydrogen peroxide. Hydrogen peroxide, in the presence of peroxidase (POD) allows the oxidative condensation of 3-methyl-N-ethyl-N-(B-hydroxyethyl)-aniline (MEHA) with 4- aminophenazone to form the final reaction product, which is a purple coloured adduct. This can be measured colorimetrically at 550 nm. The results are correct to within 1.1%. The reactions of this assay are listed below:
\[ ACS \]
Free fatty acids + ATP + CoA \arrow{\rightarrow} \text{Acyl-CoA + AMP + PPI} \\

\[ ACOD \]
Acyl-CoA + O_2 \arrow{\rightarrow} 2, 3\text{-trans-Enoyl- CoA + H}_2\text{O}_2 \\

\[ POD \]
2H_2O_2 + 4\text{-aminophenazone + MEHA} \arrow{\rightarrow} \text{Final Reaction Product + 3H}_2\text{O} \\

2.12.3 Plasma Triglyceride.

Plasma TG levels were measured using a colorimetric enzymatic kit from Roche Diagnostics GmbH (Mannheim, Germany) corrected for free glycerol concentration. The preliminary reaction removes free glycerol from the sample prior to the hydrolysis of TG. This is followed by hydrolysis of the TG to glycerol by using a lipase. Free and released glycerol is detected by an enzymatic colorimetric reaction utilizing glycerol kinase and glycerol 3-phosphate oxidase. The reactions were as follows:

Preliminary Reaction:

\[ Glycerol Kinase \]
Free glycerol + ATP \arrow{\rightarrow} \text{Glycerol 3-phosphate + ADP} \\

\[ Glycerol 3\text{-phosphate oxidase} \]
Glycerol 3-phosphate + O_2 \arrow{\rightarrow} \text{Dihydroxyacetonephosphate + H}_2\text{O}_2 \\

\[ Peroxidase \]
H_2O_2 + 4\text{-chlorophenol} \arrow{\rightarrow} \text{oxidation product}
**Assay Reaction:**

*Lipase*

\[ \text{TG} + 3\text{H}_2\text{O} \rightarrow \text{Glycerol} + \text{Fatty Acids} \]

*Glycerol Kinase*

\[ \text{Glycerol} + \text{ATP} \rightarrow \text{Glycerol 3-phosphate} + \text{ADP} \]

*Glycerol 3- phosphate oxidase*

\[ \text{Glycerol 3-phosphate} + \text{O}_2 \rightarrow \text{Dihydroxyacetone phosphate} + \text{H}_2\text{O}_2 \]

*Peroxidase*

\[ \text{H}_2\text{O}_2 + 4\text{-chlorophenazone} + 4\text{-chlorophenol} \rightarrow 4\text{-}(p\text{-benzoquilone})\text{-monooiminol-phenazone (4-PBMP)} + 2\text{H}_2\text{O} + \text{HCl} \]

The absorbance of 4-PBMP at 500 nm determines the amount of glycerol liberated from TG. The coefficient of variation for this assay was \( \sim 1.4\% \).

**2.12.4 Plasma Glucose Determination.**

Plasma glucose concentrations were measured by the glucose oxidase methods using a Beckman Glucose Analyzer II (Beckman, Fullerton, USA). A 10 µl sample of plasma containing D-glucose is pipetted into a solution containing oxygen and glucose oxidase. The glucose reacts with oxygen in the following reaction catalyzed by glucose oxidase:

\[ \text{D-glucose} + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{gluconic acid} + \text{H}_2\text{O}_2 \]

In the reaction, oxygen is used at the same rate of glucose to form gluconic acid. A polarographic oxygen sensor is used to detect oxygen consumption, which is directly proportional to the glucose concentration in the sample. Results are obtainable within 30 seconds following sample addition. Plasma sample was re-analyzed until repeated measurements were within a difference of 3 mg/dl. The analyzer was calibrated before use and frequently during the
experiment with the 150/50 glucose/urea nitrogen standard (Beckman, Fullerton, USA) that
accompanied each kit.

2.13 Statistical Analysis

Values are expressed as mean ± SEM. Statistical analysis was performed using analysis of variance (ANOVA) to test for significant differences between groups and the post hoc comparisons were performed with Tukey’s t-test. Statistical calculations were performed using SAS software (Statistical Analysis System, Cary, NC). Significance was accepted at $P<0.05$. 
Study 1

*Insulin Inhibits and Oral Sucrose Increases Neointimal Growth After Arterial Injury in Rats*

Modified from:


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3.1 Abstract

**Background/Aims:** In our previous studies, rats on insulin treatment (5U/day) and oral glucose to avoid hypoglycemia had reduced neointimal growth after arterial injury. However, plasma glucose in the insulin- treated rats was lower than normal and the effect of oral glucose remained undetermined. In this study the effects of insulin, in the presence of normal plasma glucose levels, and oral glucose or sucrose were investigated in the same model. **Methods:** Rats were divided into six groups: 1) control implants and tap water; 2) insulin implants (5U/day) and oral glucose+i.p. glucose to avoid any glucose lowering; 3) insulin implants (4U/day) and oral glucose; 4) insulin implants (4U/day) and oral sucrose; 5) control implants and oral glucose; and 6) control implants and oral sucrose. **Results:** Insulin treatment at both doses reduced neointimal area \(P<0.001\) 14 days after injury in rats receiving oral glucose but not in those receiving oral sucrose. Oral glucose, without insulin, had no effect on neointimal formation whereas oral sucrose increased neointimal growth \(P<0.05\). Oral sucrose \(P<0.05\) but not oral glucose decreased insulin sensitivity measured with hyperinsulinemic clamps. **Conclusions:** (1) Insulin decreases neointimal growth after arterial injury independent of glucose- lowering or oral glucose administration and (2) oral sucrose *per se* affects neointimal growth.
3.2 Introduction

Metabolic syndrome is a common disorder characterized by the development of a cluster of abnormalities which include insulin resistance, compensatory hyperinsulinemia, glucose intolerance (type 2 diabetes, impaired glucose tolerance, or impaired fasting glucose), central obesity, dyslipidemia, hypertension, and microalbuminuria\textsuperscript{353}. The metabolic syndrome affects up to 25% of the population\textsuperscript{353} and is associated with an increased risk of atherosclerosis and restenosis\textsuperscript{354} in part because of insulin resistance/ hyperinsulinemia \textit{per se} \textsuperscript{124, 188}.

In the metabolic syndrome, hyperinsulinemia and insulin resistance coexist, therefore it is unclear which is the main risk factor for atherosclerosis and restenosis. In our previous study, we investigated the effect of insulin on neointimal hyperplasia after balloon catheter injury, a model of restenosis. Normal rats on insulin treatment (5U/day) and given a control diet had significantly reduced neointimal growth at 14 days after arterial injury\textsuperscript{133}. However, the beneficial effect of insulin was diminished after a high fat diet, which induces insulin resistance similar to metabolic syndrome. The decrease in neointimal area induced after insulin treatment in rats fed the control diet occurred despite an increase in neointimal cell proliferation\textsuperscript{133}. However, one limitation of the study was that the plasma glucose levels in the insulin- treated rats were lower than normal, and it has been shown in previous studies that hypoglycemia induced by insulin can decrease DNA synthesis in the rat aorta up to four days after arterial injury\textsuperscript{215}. Although in our study the plasma glucose levels were not in the hypoglycemic range, the effect of insulin was not studied in the presence of normal plasma glucose.

In another study, we investigated the effects of insulin on neointimal growth after balloon catheter injury of the carotid artery in rats; however a lower dose of insulin was used. We have shown that insulin treatment (3U/day) in normal rats given oral glucose and standard diet, did not significantly decrease neointimal area at 14 days after arterial injury but decreased neointimal area at 28 days after arterial injury\textsuperscript{134}. Taken together, the results of the two studies suggest that
the action of insulin on neointimal growth may be dose-dependent, however it is also possible that in the first study neointimal growth decreased significantly because of glucose lowering rather than the higher insulin dose.

A common limitation of the two studies is that the insulin-treated rats were given oral glucose to help maintain plasma glucose levels within the normal range and the effect of oral glucose was not assessed. It is theoretically possible that the decrease in neointimal growth is caused by the oral glucose rather than insulin. Glucose intake orally stimulates gastrointestinal hormones, such as GLP-1 and GIP, whose receptors are present in vascular cells. GLP-1 has been reported to induce vasodilation\textsuperscript{355} and improve endothelial function\textsuperscript{356} whereas GIP was found to affect portal venous and hepatic arterial blood flow in dogs\textsuperscript{357}. Thus these hormones can have vascular actions, although their effect on neointimal growth has not been investigated.

The purpose of this study was to investigate the effects of insulin treatment at the higher dose of 5U/day on neointimal growth after arterial injury, while maintaining the blood glucose levels within the normal physiological range. We have also determined the effect of oral glucose, without insulin treatment, on neointimal growth after arterial injury. In addition, we wished to investigate the effect of oral sucrose alone and in combination with insulin, as a recently published study found that neointimal growth was actually increased in insulin-treated rats after balloon catheter injury while normal plasma glucose levels were maintained with 10% oral sucrose\textsuperscript{219}. This is in contrast to our studies, where we used 40% glucose in water to maintain plasma glucose and decreased neointimal growth. Sucrose contains fructose, which is a known inducer of insulin resistance\textsuperscript{220}, thus we speculate that sucrose alone (the effect of which was not evaluated in the previous study) may increase neointimal growth.
3.3 Materials and Methods

3.3.1 Animal Models

Male Sprague-Dawley rats weighing between 350-400g were used. The rats were divided into six groups: 1) rats with blank implants and access to tap water (Control = C); 2) rats with insulin implant (5U/day) and access to glucose in their drinking water as a 40% (w/v) solution (Insulin (5U/day) + 40% oral glucose = I5G) but with i.p. glucose infusion (25%) to avoid any glucose lowering and correspond to Kim et al\textsuperscript{133}; 3) rats with insulin implant (4U/day) and access to glucose in their drinking water as a 40% (w/v) solution (Insulin (4U/day) + 40% oral glucose = I4G) to compare to Foster et al\textsuperscript{219}; 4) rats with insulin implant (4U/day) and access to sucrose in their drinking water as a 10% (w/v) solution as in Foster et al\textsuperscript{219} (Insulin (4U/day) + 10% oral sucrose = I4S); 5) rats with blank implants and access to glucose in their drinking water as a 40% (w/v) solution (Control-Glucose = CG); and 6) rats with blank implants and access to sucrose in their drinking water as 10% (w/v) solution (Control-Sucrose = CS). The dose of insulin at 5U/day resulted in plasma insulin levels similar to that observed in Zucker fatty rats.

The animals were maintained as described in the General Methods section 2.1. The rats were implanted with either insulin-releasing implants (5U/day or 4U/day; LinShin Canada Inc.) or blank implants two days before balloon angioplasty as described in the General Methods section 2.2.1.

3.3.2 Surgical Procedures

Refer to the General Methods section 2.2.4 for details regarding surgical procedures. Rats were anesthetized and the LCCA was injured with a balloon catheter to denude the vessel of endothelium. After balloon injury, an intraperitoneal catheter was inserted for infusion of 25% glucose to maintain normal blood glucose levels (control rats received continuous infusion of saline) after surgery (when rats do not eat or drink) and the rate of infusion was adjusted
according to frequent glycemic determination (every 2 hours). After recovery from surgery the insulin (5U/day)-treated rats still received continuous glucose infusion for 14 days to ensure the blood glucose levels remained in the normal range and the infusion rate was monitored on a daily basis, whereas the control rats received saline infusion. In control rats we compared those receiving saline infusion via the i.p. catheter to rats without the i.p. catheter and found no difference in neointimal area ($P>0.05$) (Table 3.1). Therefore data for these animals was pooled as a single control group (C). In the rats receiving insulin at 4U/day, an i.p. catheter for glucose infusion was not used. Oral glucose or sucrose was used alone to maintain plasma glucose levels within the normal range.

3.3.3 Vessel and Blood Sample Collection

Two weeks after arterial injury the rats were sacrificed where approximately half of the rats were sacrificed in the fed state and half in the fasted state in order to obtain blood samples in different metabolic states. Under general anesthesia, blood samples were collected via cardiac puncture for insulin, FFA, and TG measurements. Rats were then sacrificed via anesthetic overdose and subjected to retrograde perfusion through the abdominal aorta with Krebs-Ringer solution and then with 10% formalin at physiologic pressure (110-120mmHg). The carotids were removed, immersed in 10% formalin for >24h, and embedded in paraffin (refer to the General Methods section 2.3.1 for details).

3.3.4 Metabolic Parameters

Blood glucose levels were measured daily either in the fasted or fed state as previously described in General Methods section 2.3. Assays for plasma insulin, FFA, and TG were performed as described in the General Methods section 2.12 of this thesis.
3.3.5 Histomorphometry/Matrix Staining

Neointimal and medial cross-sectional areas were measured on hematoxylin and eosin stained slides and analyzed using a computer-assisted morphometric system (Simple PCI, Compix Inc). Neointimal cell proliferation was measured 14 days after carotid balloon injury with an anti-BrdU antibody. Refer to the General Methods section 2.5.2 for a detailed description of staining and analysis.

3.3.6 Vessel Cannulation

Three days before the hyperinsulinemic-euglycemic clamp, rats were anesthetized with isoflurane and indwelling catheters were inserted into the right internal jugular vein for infusion and the LCCA for blood sampling, as described in the General Methods section 2.2.6.

3.3.7 Hyperinsulinemic-Euglycemic Clamp

After an overnight fast, insulin (5mU/kg.min) and glucose (variable rate) were infused through the jugular vein for 2 hours to maintain euglycemia while blood samples were taken from the LCCA for plasma glucose measurements. Refer to the General Methods section 2.10 for a detailed description of the clamp procedure.

3.3.8 Statistical Analysis

Values are expressed as mean ± SEM. Statistical analysis was performed using analysis of variance (ANOVA) to test for significant differences between groups and the post hoc comparisons were performed with Tukey’s t-test. Significance was accepted at $P<0.05$.

3.4 Results

3.4.1 Metabolic Parameters

Daily food intake, caloric intake, body weight change, as well as the fasting and fed plasma glucose levels was measured over the treatment period. Food intake was significantly less in all rats given oral glucose compared to the control group (C: 24±1g/day; I5G: 9±2g/day; I4G:
18±1g/day; I4S: 22±1g/day; CG: 14±2g/day; CS: 21±1g/day, P<0.01 I4G vs. C, P<0.001 I5G and CG vs. C) because of the 40% glucose in water. Average daily caloric intake was not significantly different among groups (C: 112±5kcal/day; I5G: 111±9kcal/day; I4G: 137±2kcal/day; I4S: 133±8kcal/day; CG: 101±9kcal/day; CS: 120±5kcal/day). The change in body weight (data not shown) was also not significantly different among groups. Neither fasting nor fed plasma glucose (Table 3.2) was different between control and insulin- treated rats receiving oral glucose; whereas fasting plasma glucose was greater in rats receiving oral sucrose alone (P<0.01). However, fasting plasma glucose was lower in insulin- treated rats receiving oral sucrose (P<0.001), suggesting that the 10% oral sucrose given to the insulin- treated rats was inadequate to maintain normal plasma glucose levels, despite insulin resistance, since these rats received less total glucose than the insulin- treated rats receiving 40% oral glucose.

Fasting and fed levels of plasma insulin, TG, and FFA were measured at 14 days after arterial injury. FFA and TG levels were assessed because dyslipidemia is present in the metabolic syndrome and we have shown they have an effect on neointimal thickness. As expected, the fasting insulin levels were greater in all insulin- treated groups than in the control groups (P<0.001) (Table 3.3). The fasting TG levels tended to be higher in the control-sucrose group (P<0.10) (Table 3.3). The fasting FFA levels in the insulin- treated rats receiving oral glucose were significantly decreased (P<0.05), whereas in the insulin- treated rats receiving oral sucrose FFA only tended to decrease (P<0.10) (Table 3.3). In the fed state, the insulin levels were greater in the insulin- treated groups than in all other groups (P<0.05), as expected (Table 3.4). The control-sucrose group tended to have elevated fed TG levels (P<0.10) (Table 3.4). The fed FFA levels were not significantly changed in any group (Table 3.4).
3.4.2 Morphometric Measurements

Figure 3.1 shows representative cross-sections of neointimal formation 14 days after balloon catheter injury of the carotid artery in all groups. Insulin treatment at a dose of 5U/day and 4U/day decreased neointimal thickening in rats receiving oral glucose compared to the control rats and those receiving oral glucose alone (*P*<0.001) (Figures 3.1, 3.2A). In contrast, rats receiving insulin treatment at 4U/day and oral sucrose had a similar neointimal area to control rats or those receiving oral glucose alone. Oral glucose alone did not have any effect on neointimal thickening versus control, however oral sucrose increased neointimal thickening (*P*<0.05) (Figures 3.1, 3.2A). We also calculated the ratio of neointimal to medial thickness, which confirm our neointimal area results (Figure 3.2B). The calculated lumen area was significantly higher in both the 5U/day and 4U/day insulin-treated groups receiving oral glucose, 0.24 ± 0.02 mm² and 0.22 ± 0.02 mm² respectively, compared to control, 0.15 ± 0.02 mm² (*P*<0.05) whereas the lumen area in the insulin (4U/day)-treated rats receiving oral sucrose (0.18 ± 0.03 mm²) and the control-glucose group (0.17 ± 0.02 mm²) were not significantly different than control. Lumen area had a slight tendency to decrease in the control-sucrose group (0.12 ± 0.01 mm²) compared to control (*P*<0.20). The medial area was similar among all groups. The external elastic lamina perimeter for the rats given oral sucrose had a very modest increase (*P*<0.05) (Figures 3.3A, 3.3B).

Neointimal cell proliferation, evaluated as % BrdU incorporation, was increased to a similar degree in the insulin-treated groups and was greater than control (*P*<0.01) (Figure 3.4). Neointimal cell proliferation in the control-glucose and control-sucrose group was similar to control. The total number of cells was counted in the neointima and was significantly decreased in both insulin-treated groups receiving oral glucose (*P*<0.05) (Table 3.5). Neointimal cell density was calculated as the total number of cells divided by neointimal cross-sectional area.
Neointimal cell density tended to be greater in the insulin- treated rats receiving oral glucose compared to control \( (P<0.10) \) (Table 3.5).

### 3.4.3 Insulin Sensitivity Measurements

To test for the possibility that the sucrose- fed rats were insulin resistant and subsequently insulin was not able to exert its inhibitory effect on neointimal growth, we used a hyperinsulinemic- euglycemic clamp to measure insulin sensitivity after 16 days (equivalent to 2 days before and 14 days after carotid balloon injury) of treatment with oral glucose or oral sucrose. A 5 mU/kg.min insulin infusion rate was chosen to assess half- maximum glucose disposal (glucose infusion rate). The glucose infusion rate during the last 30 minutes of a 2 hour hyperinsulinemic- euglycemic clamp is an indication of whole body insulin sensitivity. Plasma insulin levels during the clamp were not significantly different (C: 699±41pM; CG: 702±27pM; CS: 812±53pM). The glucose infusion rate was unchanged in rats receiving oral glucose treatment compared to those receiving tap water \( (P>0.05) \) (Figure 3.5), indicating that oral glucose did not affect insulin sensitivity. However, oral sucrose significantly decreased the glucose infusion rate \( (P<0.05) \), suggesting that indeed oral sucrose induced insulin resistance in this model.

### 3.5 Discussion.

In the present study the \textit{in vivo} effects of insulin, without changes in glycemia, and oral glucose or sucrose on neointimal formation in the rat carotid balloon injury model were investigated. Insulin treatment combined with oral glucose but not oral sucrose decreased neointimal area after injury. Oral glucose alone had no such effect, however, oral sucrose had opposite effects to those of insulin on neointimal area, which may be attributed to a decrease in insulin sensitivity.
3.5.1 Effect of insulin and oral glucose.

In the fasted state, FFA levels of the I5G and I4G group were lower than the control group. This was expected since insulin prevents lipolysis by inhibiting hormone sensitive lipase\textsuperscript{358} and suggest that these rats remained insulin sensitive. In the current study, normal plasma glucose levels, not significantly different from control, were achieved in both the fasted and fed states in the insulin (5U/day)- treated group by infusing glucose i.p. and by providing glucose in the drinking water. At the lower dose of insulin (4U/day) oral glucose was sufficient to maintain normal plasma glucose levels. The insulin treatment in rats receiving oral glucose resulted in a decrease in neointimal thickness despite an increase in neointimal cell proliferation, whereas oral glucose alone had no effect. Also, the increase in lumen area with insulin was not a result of outward remodeling since the external elastic lamina perimeter measured in these groups was the same. Taken together, these data indicate that insulin treatment reduces neointimal growth after arterial injury independent of oral glucose administration and confirms that the decrease in neointimal thickness observed in Kim et al\textsuperscript{133} was due to a direct effect of insulin and not to an insulin- induced decrease in plasma glucose. The extent of the percentage decrease was also comparable to Kim et al\textsuperscript{133}, even though our absolute neointimal thickness is greater than in the previous study, since a different experimenter was performing the surgery.

Also notable and in accordance with our previous study\textsuperscript{133} was the effect of insulin to increase neointimal cell proliferation even though neointimal area was decreased. This suggests that insulin may have both growth- promoting and vasculoprotective effects, however in our conditions the vasculoprotective effect of insulin to decrease neointimal area prevailed. An inhibitory effect on VSMC migration or stimulation of apoptosis could explain why the total number of neointimal cells did not increase with insulin despite the increase in proliferation. We have shown that a lower dose of insulin does inhibit cell migration without affecting apoptosis\textsuperscript{134}. Furthermore, in the present study insulin + oral glucose also tended to increase
neointimal cell density despite decreasing total neointimal cell number, suggesting that insulin inhibits ECM accumulation, which we have observed previously. A decrease in ECM accumulation in addition to insulin’s inhibition of cell migration would also result in a decrease in neointimal growth.

3.5.2 Effect of insulin and oral sucrose.

We also examined the effect of oral sucrose alone and in combination with insulin on neointimal growth since insulin was shown to increase neointimal hyperplasia by Foster et al. in rats where 10% sucrose water was used to maintain normoglycemia. We found that in insulin-treated rats receiving oral sucrose, the inhibitory effect of insulin on neointimal growth was not observed while oral sucrose alone increased neointimal thickness after arterial injury. Interestingly, however, oral sucrose did not block the effect of insulin to increase neointimal cell proliferation suggesting the development of selective insulin resistance.

Sucrose is a disaccharide of glucose and fructose. Both high-sucrose and high-fructose feeding are commonly used to induce insulin resistance in rats and studies have shown that fructose is the nutrient mediator of sucrose-induced insulin resistance. Glucose feeding however, did not affect insulin sensitivity. The oral sucrose group in our study had elevated fasting plasma glucose levels, indicating that these rats were insulin resistant, which was confirmed with the hyperinsulinemic-euglycemic clamp as oral sucrose decreased insulin sensitivity compared to the tap water and oral glucose groups. In addition, the oral sucrose group tended to have increased fasting and fed TG levels, which are also associated with insulin resistance and are observed after sucrose feeding as fructose bypasses the glucokinase checkpoint and also the phosphofructokinase checkpoint in the liver, immediately entering glycolysis and fatty acid synthesis. Sucrose-induced protein glycation may also be involved as advanced glycation endproducts (AGEs) damage endothelial cells and ECM proteins, which could promote neointimal growth or explain the increased external elastic perimeter (an
indication of vessel remodeling). Therefore, it seems that in Foster et al., the oral sucrose given to the insulin-treated rats reduced insulin sensitivity, thus blocking insulin’s action to inhibit neointimal growth. However, insulin sensitivity at the level of the vessel was not assessed. Future studies could measure markers of insulin sensitivity, for example serine phosphorylation of IRS-1, via Western blot analysis. Why in Foster’s study insulin + oral sucrose actually increased neointimal growth whereas in our study it only failed to decrease it compared to control is not clear. The rats in Foster’s study are older than ours and perhaps more insulin resistant, thus it is possible that the effect of insulin was enough to counteract the effect of oral sucrose in our study.

In conclusion, we demonstrate that insulin treatment reduces neointimal growth at 14 days after arterial injury due to an effect of insulin and not because of insulin-induced glucose lowering or oral glucose ingestion, and that oral sucrose should not be used to maintain normoglycemia while giving insulin because it increases neointimal growth. Thus, these results further support a vascular protective effect of insulin in vivo.
Table 3.1. Neointimal formation with or without an i.p. catheter. Cross-sectional areas of the intima of carotid arteries from control rats with an i.p. catheter and control rats without an i.p. catheter measured at 14 days after vessel injury.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control With i.p. Catheter (n=5)</td>
</tr>
<tr>
<td>Intimal Area (mm²)</td>
<td>0.23±0.02</td>
</tr>
</tbody>
</table>

Values are means ± SEM. No significant differences were found.
Table 3.2. Daily fasting and fed plasma glucose levels over the treatment period.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (n=10)</td>
<td></td>
</tr>
<tr>
<td>I5G (n=9)</td>
<td></td>
</tr>
<tr>
<td>I4G (n=8)</td>
<td></td>
</tr>
<tr>
<td>I4S (n=8)</td>
<td></td>
</tr>
<tr>
<td>CG (n=10)</td>
<td></td>
</tr>
<tr>
<td>CS (n=10)</td>
<td></td>
</tr>
<tr>
<td><strong>Fasting Plasma Glucose (mM)</strong> †</td>
<td></td>
</tr>
<tr>
<td>4.9 ± 0.1</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td>4.5 ± 0.5</td>
<td>3.2 ± 0.3***</td>
</tr>
<tr>
<td>5.1 ± 0.2</td>
<td>6.3 ± 0.1**</td>
</tr>
<tr>
<td><strong>Fed Plasma Glucose (mM)</strong> ‡</td>
<td></td>
</tr>
<tr>
<td>6.1 ± 0.1</td>
<td>6.1 ± 0.2</td>
</tr>
<tr>
<td>6.1± 0.2</td>
<td>5.9± 0.4</td>
</tr>
<tr>
<td>6.1 ± 0.1</td>
<td>6.2 ± 0.1</td>
</tr>
</tbody>
</table>

C=Control; I5G=Insulin (5U/day) + oral glucose; I4G=Insulin (4U/day) + oral glucose; I4S=Insulin (4U/day) + oral sucrose; CG=Oral Glucose; CS=Oral Sucrose. Data are expressed as means ± SEM. **P<0.01, ***P<0.001 vs. C. Glucose levels were measured daily for 14 days. † Data are means ± SEM of individual average glucose levels taken over the treatment period after an overnight 16 hour fast. ‡ Data are means ± SEM of individual average glucose levels taken over the treatment period.
Table 3.3. Fasting plasma levels of insulin, triglycerides, and free fatty acids at 14 days after arterial injury.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C (n=5)</td>
</tr>
<tr>
<td>Insulin (pM)</td>
<td>82±8</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>0.72±0.16</td>
</tr>
<tr>
<td>Free Fatty Acids (uEq/L)</td>
<td>632±118</td>
</tr>
</tbody>
</table>

C=Control; I5G=Insulin (5U/day) + oral glucose; I4G=Insulin (4U/day) + oral glucose; I4S=Insulin (4U/day) + oral sucrose; CG=Oral Glucose; CS=Oral Sucrose. Data are expressed as means ± SEM and represent values taken at sacrifice. *P<0.05, **P<0.001 vs. C.
Table 3.4. Fed plasma levels of insulin, triglycerides, and free fatty acids at 14 days after arterial injury.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C (n=5)</td>
</tr>
<tr>
<td><strong>Insulin (pM)</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>170±26</td>
</tr>
<tr>
<td><strong>Triglycerides (mM)</strong></td>
<td>1.23±0.19</td>
</tr>
<tr>
<td><strong>Free Fatty Acids (uEq/L)</strong></td>
<td>230±14</td>
</tr>
</tbody>
</table>

C=Control; I5G=Insulin (5U/day) + oral glucose; I4G=Insulin (4U/day) + oral glucose; I4S=Insulin (4U/day) + oral sucrose; CG=Oral Glucose; CS=Oral Sucrose. Data are expressed as means ± SEM and represent values taken at sacrifice. *P<0.05, **P<0.001 vs. C.
Table 3.5. Intimal cell number and cell density per unit area measured at 14 days after vessel injury.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (n=10)</td>
<td></td>
</tr>
<tr>
<td>15G (n=7)</td>
<td>1276±93</td>
</tr>
<tr>
<td>14G (n=8)</td>
<td>914±47*</td>
</tr>
<tr>
<td>14S (n=8)</td>
<td>864±47*</td>
</tr>
<tr>
<td>CG (n=9)</td>
<td>1238±77</td>
</tr>
<tr>
<td>CS (n=10)</td>
<td>1308±162</td>
</tr>
<tr>
<td></td>
<td>1440±113</td>
</tr>
</tbody>
</table>

Intimal Cell Number (cells/mm²)

|                    | C=Control; I5G=Insulin (5U/day) + oral glucose; I4G=Insulin (4U/day) + oral glucose; I4S=Insulin (4U/day) + oral sucrose; CG=Oral Glucose; CS=Oral Sucrose. Data are expressed means ± SEM. *P<0.05 vs. C. #P<0.05, ##P<0.01 vs. CG. |
Figure 3.1. The effect of insulin, oral glucose, and oral sucrose on neointimal formation. Representative photomicrographs of cross-sections taken at 14 days after carotid injury (x400). L=Lumen; I=Intima; M=Media.
Figure 3.2. The effect of insulin, oral glucose, and oral sucrose on intimal area. (A) Cross-sectional areas of the intima of carotid arteries measured at 14 days after carotid injury (C: n=10; I5G: n=9; I4G: n=8; I4S: n=8; CG: n=10; CS: n=10). (B) Ratio of intima: medial area. Values are means ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs. C. ###P<0.01, ####P<0.001 vs. CG. §P<0.001 vs. CS.
Figure 3.3. The effect of insulin, oral glucose, and oral sucrose on medial area and external elastic lamina perimeter. Cross-sectional areas of the (A) media and (B) external elastic lamina of carotid arteries measured at 14 days after carotid injury (C: n=10; I5G: n=9; I4G: n=8; I4S: n=8; CG: n=10; CS: n=10). Values are means ± SEM. *P<0.05, **P<0.01 vs. C.
Figure 3.4. The effect of insulin, oral glucose, and oral sucrose on cell proliferation. Cell proliferation expressed as % BrdU-labeled cells in the intima at 14 days after carotid injury (C: n=10; I5G: n=7; I4G: n=8; I4S: n=8; CG: n=9; CS: n=10). Values are means ± SEM. **P<0.01, ***P<0.001 vs. C. *P<0.05 vs. CG. §P<0.001 vs. CS.
Figure 3.5. The effect of oral glucose and oral sucrose on whole body insulin sensitivity. Whole body insulin sensitivity is indicated by the glucose infusion rate during the last 30 min of the 2h hyperinsulinemic- euglycemic clamp (C: n=10; CG: n=9; CS: n=8). Values are expressed as mean ± SEM. *P<0.05 vs. C.
Study 2

*The Vasculoprotective Effects of Insulin are Nitric Oxide Synthase-Dependent*

The data presented in Study 2 are currently unpublished except for Table 4.3 and Figure 4.4A-F, which are published in and modified from:


*Permission to reproduce portions of the above manuscript has been obtained from the copyright owner, American Heart Association.*
4.1 Abstract

**Background/Aims:** Metabolic syndrome, characterized by insulin resistance, is a risk factor for atherosclerosis and restenosis after percutaneous revascularization procedures. Insulin has protective effects on the endothelium by stimulating nitric oxide (NO) production and endothelial nitric oxide synthase (eNOS) expression but also has mitogenic effects on vascular smooth muscle cells (VSMC) *in vitro*. Furthermore, NOS inhibition has been reported to abolish the effect of insulin to inhibit VSMC migration *in vitro*. We have previously shown that insulin decreases neointimal growth and cell migration to the neointima after carotid angioplasty in normal rats. Since insulin can stimulate NOS and NO can decrease neointimal growth, we investigated the consequences of NOS inhibition, with the *in vivo* inhibitor L-NAME, on the inhibitory effects of insulin.

**Methods:** Rats were given subcutaneous implants and divided into four groups: 1) control; 2) insulin (3U/day); 3) insulin+L-NAME (2mg/kg/day); and 4) control+L-NAME. Treatment began three days before arterial (carotid or aortic) balloon catheter injury and was continued post-injury. **Results:** Insulin decreased both neointimal area (*P*<0.05) and cell migration (*P*<0.001), and increased re-endothelialization (*P*<0.05). The addition of L-NAME abolished insulin’s effect on neointimal growth, cell migration, and also re-endothelialization. Furthermore, insulin increased expression of VSMC differentiation markers (*P*<0.05) and the number of circulating progenitor cells (*P*<0.05). eNOS protein expression tended to increase with insulin treatment (*P*=0.08). **Conclusions:** These results show that insulin has a protective effect on both the VSMC and endothelium *in vivo*, and that NO plays a role in mediating these effects.
4.2 Introduction

Type 2 diabetes and metabolic syndrome are risk factors for atherosclerotic CVD, partly because of associated abnormalities such as hypertension, dyslipidemia, glucose intolerance or overt hyperglycemia, and partly because of the atherogenic risk of insulin resistance and/or hyperinsulinemia per se. There is evidence for both growth promoting and protective vascular effects of insulin. For example, insulin increases proliferation of VSMCs in vitro. However, insulin also stimulates endothelial cell production of NO, which inhibits VSMC growth and migration, and has anti-inflammatory effects.

In addition to the increased risk for atherosclerosis, patients with diabetes and metabolic syndrome have an increased risk for restenosis after PTCA and stenting. After balloon angioplasty, neointimal injury results in endothelial denudation, followed by platelet activation and secretion of growth factors from platelets and damaged VSMCs. Subsequently, there is VSMC migration and proliferation, and ECM deposition resulting in neointimal growth and lumen narrowing.

Although the effect of insulin on restenosis remains a point of debate, we have shown that insulin treatment is protective and decreases neointimal formation after arterial injury. In addition, we observed that insulin, without changes in glycemia, decreases cell migration and accelerates re-endothelialization, resulting in decreased neointimal growth.

Early re-endothelialization of the injured vessels inhibits lesion development, facilitates vascular repair, and improves long term vessel patency. Vascular endothelial cells produce the vasodilator molecule NO, which has significant protective effects on several steps of the restenosis process as outlined in the Introduction section. For example, increased eNOS expression via gene transfer diminishes neointimal formation after arterial injury and eNOS deficiency results in greater neointimal growth after cuff injury. Furthermore treatment of
ApoE knockout mice with AVE9488, a compound that increases eNOS mRNA and protein expression, reduced neointimal formation after cuff-injury\textsuperscript{366}. The primary role of iNOS on neointimal formation is less clear, with studies indicating that it accelerates restenosis\textsuperscript{154}, and others indicating that it decreases it\textsuperscript{156, 157}. More recently, adenoviral-medicated gene delivery of eNOS and iNOS following endothelial injury in the rabbit carotid artery showed that both eNOS and iNOS reduced neointimal formation, however eNOS gene delivery also accelerated re-endothelialization whereas iNOS gene delivery actually inhibited re-endothelialization, highlighting a possible advantage of eNOS\textsuperscript{367}.

Insulin activates and upregulates eNOS via PI3K and Akt\textsuperscript{235, 236}. Insulin has also been reported to upregulate iNOS\textsuperscript{239} and enhance NO-induced guanylate cyclase activity in VSMCs\textsuperscript{240}. Furthermore, insulin’s inhibitory effect on VSMC migration \textit{in vitro} was abolished by NOS inhibitors\textsuperscript{272}. Thus, NOS activation represents a likely mechanism for insulin to exert its inhibition of neointimal formation. Therefore, the objective of our study is to investigate the consequences of NOS inhibition on the effects of insulin.

\section*{4.3 Materials and Methods}

\subsection*{4.3.1 Animal Models}

Four groups of male Sprague-Dawley rats weighing 400-450g and fed normal rat chow were studied: 1) control (C); 2) insulin (3U/day; s.c. capsule (bovine insulin))-treated (LinShin Inc.) (I); 3) insulin + NG-nitro-L-arginine methyl ester (L-NAME) (2mg/kg/day) (I+L2); and 4) control + L-NAME (C+L2). Insulin was given at 3U/day, which is lower than the dose used in Study 1, as 40% oral glucose was previously shown to maintain plasma glucose levels similar to control without the additional i.p. glucose infusion\textsuperscript{134}. This dose of L-NAME has been previously shown to inhibit NO production in the rat aorta, based on vessel cGMP assays\textsuperscript{368}, without affecting blood pressure\textsuperscript{369}. All insulin-treated rats were given 40% glucose in drinking water to
maintain normoglycemia, which we have found in Study 1 to have no effect on neointimal formation. The animals were maintained as described in the General Methods section 2.1.

4.3.2 Surgical Procedures

Three days after onset of treatment, injury to either the LCCA or the aorta of the rat was induced by means of an inflated balloon catheter\textsuperscript{133}. Refer to the General Methods section 2.2.4/2.2.5 for details regarding surgical procedures.

4.3.3 Vessel and Blood Sample Collection

The rats were sacrificed in either the fed or fasted state at various time points after injury, based on previous studies investigating the kinetics of vascular injury. Blood samples were collected by cardiac puncture under general anesthesia. Thereafter, rats were sacrificed via anesthetic overdose and the carotids were perfusion-fixed for four minutes with 10% buffered formalin at physiologic pressure (110-120mmHg). The carotids were removed, immersed in 10% formalin for >24h and embedded in paraffin. Refer to General Methods section 2.3.1 for a complete description.

4.3.4 Metabolic Parameters

Blood glucose was monitored throughout the treatment using a glucometer (see General Methods section 2.3). Plasma insulin was determined by radioimmunoassay using kits specific for rat insulin. Plasma FFA and TG were measured using specific colorimetric kits. Refer to General Methods section 2.12 for details.

4.3.5 Histomorphometry/Matrix Staining

Cell migration into the neointima was measured four days after injury and neointimal cells on the lumen surface were immunostained with an antibody against histone H1 and counted\textsuperscript{82}. Refer to General Methods section 2.5.1 for complete details. Cross-sectional areas were measured on hematoxylin and eosin stained slides at 14 and 28 days after injury. Cross-sections taken at 28 days after injury were also stained with Movat’s pentachrome or PSR dye
for elastin and collagen respectively. Refer to the General Methods section 2.5.4 for a detailed description of staining and analysis.

4.3.6 Evan’s Blue Staining

Re-endothelialized area was measured at 14 days after balloon injury and visualized with Evan’s Blue dye, which does not stain endothelial cells. Following perfusion, the thoracic aorta was dissected out and opened longitudinally where the area on the luminal surface stained with Evan’s Blue was measured (General Methods section 2.5.5). Cross-sectional areas were measured on hematoxylin and eosin stained slides from unopened sections of these aortas as described in the General Methods section 2.5.

4.3.7 Western Blot Analysis

Carotids were removed seven days after injury, homogenized, and processed as previously described in the General Methods section 2.6. Protein was separated by SDS-PAGE and analyzed with anti-SM-MHC, anti-SM22α, anti-SMC α-actin, anti-total eNOS, and anti-alpha tubulin antibodies. Refer to the General Methods section 2.8 for a detailed description.

4.3.8 Flow Cytometry

Rat blood was obtained 3 and 7 days after carotid injury in separate rats by cardiac puncture and flow cytometry was performed as in Fadini et al \textsuperscript{115} (see General Methods section 2.9 for details).

4.3.9 Blood Pressure Analysis

Systolic blood pressure was measured indirectly from the tail via the cuff technique, using the Visitech BP-2000 Blood Pressure Analysis System (see General Methods section 2.11 for details). Blood pressure was measured before treatment and throughout at 4, 14, and 28 days after arterial injury.
4.3.10 Statistical Analysis

Values are expressed as mean ± SEM. Statistical analysis was performed using analysis of variance (ANOVA) to test for significant differences between groups and the post hoc comparisons were performed with Tukey’s t-test. Significance was accepted at $P<0.05$.

4.4 Results

4.4.1 Metabolic Parameters

Daily food intake, caloric intake, body weight change, as well as the fasting and fed plasma glucose levels were measured over the treatment period. Food intake was less for insulin (I)- and I+L-treated rats (C: 29±1g/day; I: 19±1g/day; I+L2: 20±1g/day; C+L2: 29±1g/day, $P<0.001$ I and I+L vs. C), however despite this, the insulin and I+L group had greater total caloric intake compared to controls due to the 40% glucose in drinking water (C: 93±3kcal/day; I: 125±3kcal/day; I+L2: 126±2kcal/day; C+L2: 95±3kcal/day, $P<0.001$ I and I+L2 vs. C). There were no significant changes in body weight, however the insulin- and I+L2-treated rats tended to gain more weight than controls (C: 91±9g; I: 121±13g; I+L2: 114±10g; C+L2: 84±11g, $P>0.20$ I vs. C; $P>0.30$ I+L2 vs. C). Both fasting and fed plasma glucose levels taken over the treatment period were similar in all treatment groups (Table 4.1).

Fed levels of plasma insulin, TG, and FFA were measured at 28 days after arterial injury. FFA and TG levels were assessed because dyslipidemia is present in the metabolic syndrome and we have shown they have an effect on neointimal thickness. As expected, plasma insulin levels were greater in the insulin-treated groups than in all other groups ($P<0.01$) (Table 4.2). Fed TG and FFA levels were not significantly different from control.
4.4.2 Morphometric Measurements

Treatment with insulin for 28 days after balloon injury resulted in decreased neointimal area (Figure 4.1A,B) \((P<0.05)\). This effect was completely blocked by co- treatment with the NOS inhibitor L-NAME, indicating an NO- dependent effect of insulin. We also calculated the ratio of neointimal to medial thickness, which confirm our neointimal area results (Figure 4.1C). By contrast, there was no change in medial area compared to control \((C=0.15\pm0.01\text{mm}^2, \ I=0.15\pm0.01\text{mm}^2, \ I+L2=0.16\pm0.01\text{mm}^2, \ C+L2=0.16\pm0.01\text{mm}^2)\). There were no significant differences in external elastic lamina perimeter compared to control \((C=2.8\pm0.1\text{mm}, \ I=2.6\pm0.2\text{mm}, \ I+L2=3.0\pm0.1\text{mm}, \ C+L2=3.0\pm0.1\text{mm})\), indicating that outward remodeling did not likely contribute to the changes in neointimal area with treatment.

ECM accumulation is a major determinant of neointimal size. Therefore, vessel cross-sections were stained with Movat’s pentachrome for elastin or PSR for collagen to assess the ECM contribution. Insulin significantly decreased elastin accumulation by 50.7\% \((P<0.001)\) and had a slight tendency to decrease collagen accumulation by 31.8\% \((P=0.30)\) in the intima of the carotid at 28 days compared to control, and this effect was prevented by co- administration of L-NAME (Figure 4.2).

To investigate whether insulin decreased cell migration through a NOS- dependent mechanism, we measured cell migration from the media to the neointima four days after vessel injury. Insulin markedly inhibited migration by ~62\%, reducing the number of neointimal cells from 534\pm55\text{cells/mm}^2 in control rats to 204\pm55\text{cells/mm}^2 in the insulin group \((P<0.001)\) (Figure 4.3A,B). The addition of L-NAME completely abolished the effect of insulin to decrease migration and resulted in even greater cell migration than control \((I+L2=787\pm87\text{cells/mm}^2) \ (P<0.05)\).

VSMC dedifferentiation from the contractile to the synthetic migratory/ proliferative phenotype is associated with increased VSMC migration and proliferation. \textit{In vitro} studies have shown that insulin promotes VSMC differentiation\(^{370}\), therefore we investigated the \textit{in vivo} effect
of insulin on the VSMC phenotype by measuring three markers of differentiation seven days after injury (Figure 4.4A-F). Western blots revealed an increase in SM-MHC ($P<0.05$), SM22$\alpha$ ($P<0.05$), and SMC $\alpha$-actin ($P<0.01$) expression with insulin.

Since recruitment of circulating progenitor cells contributes to neointima formation$^{18}$, we used flow cytometry to detect circulating progenitor cells, which were defined by sca-1 or c-kit positivity$^{115,153}$. Insulin increased the number of circulating cells positive for sca-1 ($P<0.05$) (Table 4.3) and tended to increase the number of c-kit positive cells at 3 days after injury ($P=0.18$). There were no significant differences observed with insulin treatment for cells positive for VEGFR2, an endothelial cell marker. Only a small quantity of cells were double positive for c-kit and VEGFR2 (double positivity would indicate an endothelial progenitor cell), however there was no difference with insulin treatment. In addition, we could not detect any cells double positive for sca-1 and VEGFR2, which is similar to Fadini et al$^{115}$.

Circulating precursors also contribute to re-endothelialization and it is possible that neointimal formation is reduced by accelerated re-endothelialization. To address this, we used a model of aortic injury where re-endothelialization is more consistent and complete$^{371}$. Analysis of rat aorta specimens after Evan’s Blue staining at 14 days showed that insulin treatment accelerated re-endothelialization of the balloon-injured arterial segments by $\sim22\%$ compared to control ($P<0.05$) (Figure 4.5A,B), which was completely prevented by co-administration of L-NAME. This was accompanied by a significant reduction in neointimal thickening in the aorta after balloon injury in the insulin-treated group ($P<0.01$), which was prevented by L-NAME, (Figure 4.5C) as also observed in the carotid artery.

In an attempt to begin to address the specific NOS isoform that may be involved in insulin action, we used Western blot to measure changes in eNOS protein expression after balloon injury in the vessel. Insulin treatment tended to increase eNOS expression compared to control ($P=0.08$) (Figure 4.4G,H).
Furthermore, no changes were observed in systolic blood pressure with either insulin or L-NAME administration over the course of treatment (Figure 4.6).

4.5 Discussion

The present study focused on the in vivo effect of insulin on the kinetics of neointimal growth in the rat arterial injury model and the possible involvement of NO in insulin action. Insulin accelerated re-endothelialization and had a marked inhibitory effect on injury-induced cell migration, leading to decreased neointimal area. The inhibitory effect of insulin on cell migration and neointimal formation, as well as the stimulatory effect on re-endothelialization, was completely abolished by L-NAME. Therefore, our data provide evidence for a protective effect of insulin on vascular growth that is mediated by NO.

We did not measure neointimal cell proliferation or apoptosis after insulin treatment as we have previously found no effect of subcutaneous insulin (3U/day) treatment on proliferation or apoptosis in the same model of carotid balloon injury\textsuperscript{134}.

Insulin increases VSMC differentiation as indicated by increased VSMC differentiation markers. As mentioned above, medial VSMCs contribute to neointimal growth by dedifferentiating and migrating to the intima. Insulin could have affected migration via an effect on cell motility\textsuperscript{273} and/ or through the regulation of the medial VSMC phenotype\textsuperscript{370}. In vitro studies have implicated a PI3K- iNOS- NO- cGMP- dependent pathway for insulin to inhibit CamKinaseII activation and VSMC migration\textsuperscript{239, 273}, and promote the maintenance of a quiescent phenotype.

As previously mentioned in the Introduction section 1.7, studies have demonstrated that NO decreases the synthesis of ECM matrix components\textsuperscript{182, 184}. In agreement with these studies, we found a NO-mediated decrease in ECM accumulation after insulin treatment. This decrease in ECM production likely contributes to the decrease in neointimal area and is consistent with
insulin preventing the VSMC switch from a contractile to synthetic migratory/proliferative phenotype.

Of the three isoforms of NOS, eNOS is the most likely to be involved in insulin action as eNOS-derived NO induces vasorelaxation, inhibits leukocyte-endothelial adhesion, platelet aggregation, VSMC migration and proliferation, and increases re-endothelialization\textsuperscript{163}. As previously mentioned, upregulation of eNOS diminishes neointimal formation after arterial injury\textsuperscript{152, 372} and eNOS deficiency promotes neointimal growth after cuff injury\textsuperscript{104, 195}. Carotid balloon injury, contrary to cuff injury\textsuperscript{373}, results in complete denudation of the endothelium and re-endothelialization is seldom complete\textsuperscript{104, 374}. Since there is no endothelium after balloon injury, it might be argued that insulin-induced changes in eNOS should not be important. However, eNOS can be activated in the endothelial cells bordering the lesion to inhibit neointimal thickness and circulating EPCs expressing eNOS contribute to re-endothelialization\textsuperscript{20}. In addition, eNOS is expressed in platelets\textsuperscript{147} and insulin has a potent effect on stimulating platelet eNOS\textsuperscript{375} and NO production\textsuperscript{376}, thereby decreasing platelet adhesion and aggregation\textsuperscript{131, 377}. A reduction in the number of platelets at the site of injury would also have an additional benefit of reducing VSMC migration, as platelets have a key role in promoting VSMC migration through PDGF secretion. Consistent with our results using L-NAME, L-arginine, the precursor of NO, has been shown to inhibit neointimal formation after arterial balloon injury in normal rats\textsuperscript{378} and rabbits\textsuperscript{105}, although the specific NOS isoform activated was not addressed.

Insulin has also been reported to stimulate iNOS\textsuperscript{239, 240} in VSMCs, which do not express eNOS after injury\textsuperscript{148, 379}. However, as discussed in the introduction, the role of iNOS on neointimal formation is not clear.

Re-endothelialization begins in the endothelium (expresses eNOS) at the margin of the lesion\textsuperscript{380} and from circulating precursors\textsuperscript{20}, and is stimulated by NO\textsuperscript{166}. As previously mentioned, gene delivery of eNOS increased re-endothelialization whereas iNOS gene delivery inhibited re-
endothelialization after endothelial injury in the rabbit carotid artery, although both resulted in decreased neointimal formation\textsuperscript{367}. Furthermore, we have shown that insulin increases the number of circulating progenitor cells, which are known to express eNOS\textsuperscript{20}. Since insulin only tended to increase eNOS expression in the vessel, insulin may also increase eNOS activity as insulin is known to activate\textsuperscript{235} and upregulate\textsuperscript{236} eNOS via PI3K. In summary, insulin stimulated endothelial regeneration, tended to increase eNOS protein expression, and also suppressed neointimal thickening. Taken together, these data suggest that eNOS is the specific NOS isoform involved in insulin action to decrease neointimal formation as activation of iNOS may have likely resulted in decreased re-endothelialization. Unfortunately, studies using an eNOS or iNOS specific \textit{in vivo} inhibitor are limited as there is no specific eNOS inhibitor and studies using prolonged iNOS inhibition are not feasible due to the high cost of the inhibitors. Not much is known about the role of nNOS in the vascular system, although there is one report of induction of nNOS in VSMC after arterial injury and aggravation of neointimal formation after nNOS inhibition\textsuperscript{159}. Insulin has also been shown to upregulate nNOS in neurons\textsuperscript{238}.

Hypertension is among the cluster of disorders present in the metabolic syndrome\textsuperscript{353} and available studies have demonstrated that insulin may improve blood pressure in models of hypertension\textsuperscript{381, 382}, however in the present study blood pressure was not affected by insulin. L-NAME, at specific doses, is known to be hypertensive as it blocks NO-mediated vasodilatation. However, although not measured in this study, the dose of L-NAME used was previously shown to inhibit NO production based on vessel cGMP assays\textsuperscript{368} without affecting blood pressure\textsuperscript{369}.

In conclusion, we have shown for the first time that the effect of insulin to decrease neointimal formation after arterial injury is NO-dependent. These results provide evidence for inhibition of the growth response to vessel injury by insulin \textit{in vivo} and suggest that in metabolic syndrome insulin resistance may result in loss of vascular protection.
Table 4.1. Daily fasting and fed plasma glucose levels over the treatment period.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C (n=10)</td>
</tr>
<tr>
<td>Fasting Plasma Glucose (mM) †</td>
<td>5.0±0.2</td>
</tr>
<tr>
<td>Fed Plasma Glucose (mM) ‡</td>
<td>6.5±0.1</td>
</tr>
</tbody>
</table>

C=Control; I=Insulin; L2=L-NAME (2mg/kg/day). Data are expressed as means ± SEM. No significant differences were found compared to control. Glucose levels were measured daily for 28 days. † Data are means ± SEM of individual average glucose levels taken over the treatment period after an overnight 16 hour fast. ‡ Data are means ± SEM of individual average glucose levels taken over the treatment period.
Table 4.2. Fed plasma levels of insulin, triglycerides, and free fatty acids at 28 days after arterial injury.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C (n=7)</td>
</tr>
<tr>
<td>Insulin (pM)</td>
<td>128±16</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>1.87±0.23</td>
</tr>
<tr>
<td>Free Fatty Acids (uEq/L)</td>
<td>311±69</td>
</tr>
</tbody>
</table>

C=Control; I=Insulin; L2=L-NAME (2mg/kg/day). Data are expressed as means ± SEM and represent values taken at sacrifice. **P<0.01 vs. C.

Note: The rats used to measure neointimal formation in Figure 4.1 were randomly sacrificed in either the fed or fasting state, and as a result there are fewer rats in each treatment group in the above table compared to Figure 4.1. This may have limited the power to detect differences between treatment groups.
Table 4.3. Percentage of white blood cells stained positive for c-kit, sca-1, and VEGFR2.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Basal</th>
<th>3 days</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated/Uninjured (n=5)</td>
<td>C (n=7)</td>
<td>I (n=7)</td>
</tr>
<tr>
<td>c-kit&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.8 ± 0.6</td>
<td>3.6 ± 0.3</td>
<td>4.4 ± 0.5</td>
</tr>
<tr>
<td>sca-1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.16 ± 0.02</td>
<td>0.17 ± 0.01</td>
<td>0.26 ± 0.03&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>VEGFR2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.068 ± 0.008</td>
<td>0.049 ± 0.005</td>
<td>0.053 ± 0.006</td>
</tr>
<tr>
<td>c-kit/VEGFR2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.002 ± 0.002</td>
<td>0.003 ± 0.002</td>
<td>0.003 ± 0.002</td>
</tr>
<tr>
<td>sca-1/VEGFR2&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;†&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

C=Control; I=Insulin. Data are expressed as means ± SEM and represent values taken at sacrifice. <sup>⁎</sup><em>P</em><0.05 vs. C.

<sup>a</sup>Expressed as % of all cells defined (gated) as white blood cells.

<sup>†</sup>Note: In the Fadini et al<sup>115</sup> paper sca-1<sup>+</sup> cells separated by fluorescence-activated cell sorting also showed very little positivity for VEGFR2.
Figure 4.1. The effect of insulin and NOS inhibition on intimal area. (A) Representative photomicrographs of cross-sections taken at 28 days after carotid injury (x400). (B) Cross-sectional areas of the intima of carotid arteries measured at 28 days after injury. (C) Ratio of intimal: medial area (C: n=10; I: n=8; I+L2: n=8; C+L2: n=8). Values are means ± SEM. *P<0.05 vs. C, #P<0.05 vs. I+L2.
Figure 4.2. The effect of insulin and NOS inhibition on elastin and collagen accumulation. Representative images of staining with (A) Movat’s pentachrome to show elastin and (C) PSR to show collagen in the intima at 28 days after carotid injury. Quantitative analysis of cross-sections stained for elastin (B) or collagen (D) (C: n=10; I: n=8; I+L2: n=8; C+L2: n=8). Values are means ± SEM. ***P<0.001 vs. C, #P<0.05 vs. I+L2.
Figure 4.3. The effect of insulin and NOS inhibition on cell migration. (A) Representative photomicrographs showing cells on the lumen surface at 4 days after carotid injury (x400). (B) Quantitative analysis of cell migration (C: n=7; I: n=10; I+L2: n=8; C+L2: n=6). Values are means ± SEM. *P<0.05, ***P<0.001 vs. C, ###P<0.001 vs. I+L2.
Figure 4.4. The effect of insulin on SMC differentiation markers and eNOS expression. Representative Western blot showing (A) SM-MHC, (C) SM22α, (E) SMC α-actin, and (G) eNOS. Densitometric analysis of Western blots to detect (B) SM-MHC, (D) SM22α, (F) SMC α-actin, and (H) eNOS (C: n=7-8; I: n=7-8). Values are means ± SEM. *P<0.05, §P<0.01 vs. C, P=0.08 vs. C.
Figure 4.5. The effect of insulin and NOS inhibition on re-endothelialization. (A) Representative images of Evan’s Blue dye staining of aorta at 14 days after vessel injury. (B) Quantification of re-endothelialized area (% of total vessel area). (C) Cross-sectional areas of the intima in the aorta at 14 days after vessel injury (C: n=7; I: n=8; I+L2: n=8; C+L2: n=7). Values are means ± SEM. *P<0.05, **P<0.01 vs. C, ##P<0.01 vs. I+L2.
Figure 4.6. The effect of insulin and NOS inhibition on systolic blood pressure. Systolic blood pressure at 3 days before and 4, 14, and 28 days after carotid injury (corresponding to day 0, 7, 17, and 31 of treatment respectively). (C: n=10; I: n=8; I+L2: n=8; C+L2: n=8). Values are means ± SEM. No significant differences were found.
Study 3

Resveratrol Inhibits Neointimal Formation After Arterial Injury Through A Nitric Oxide Synthase-Dependent Mechanism

Modified from:


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5.1 Abstract

Background/Aims: Revascularization procedures used for treatment of atherosclerosis often result in restenosis. Resveratrol (RSV), a red wine polyphenol, decreases vascular smooth muscle cell (VSMC) proliferation and increases endothelial nitric oxide synthase (eNOS) expression in vitro. RSV has been shown to decrease neointimal formation after arterial injury, however our understanding of the mechanism is still incomplete. Our main objective was to address the NO- dependence of this effect. Methods: RSV (4mg/kg/day,s.c.) alone or in combination with the NOS inhibitor N-Nitro-L-arginine methyl ester (L-NAME) (2mg/kg/day,s.c.) was given to Sprague-Dawley rats beginning at 3 days before arterial (carotid or aortic) injury. Vessels (carotid or aorta) were collected at 4, 7, 14, and 28 days after injury. Results: RSV reduced neointimal formation by 50% at 28 days (P<0.05) and decreased neointimal cell proliferation by 37% at 7 days (P<0.01) after injury. RSV also inhibited cell migration by 77% at 4 days after injury (P<0.001) but did not affect re-endothelialized area at 14 days in the aortic injury model. The RSV- induced decrease in neointimal area and cell proliferation was abolished by co- treatment with L-NAME while the RSV- induced decrease in cell migration was not. Conclusions: This is the first demonstration of RSV decreasing neointimal cell proliferation and neointimal growth through a NOS- dependent mechanism. These results support a vasculoprotective role for RSV and suggest that RSV may have clinical potential in the prevention and treatment of restenosis after angioplasty.
5.2 Introduction

Vascular injury leads to pathologic repair and remodeling that involves VSMC migration and proliferation and ECM deposition, subsequently resulting in neointimal hyperplasia (reviewed in\textsuperscript{17}). Endothelial cell loss is a major contributing factor to the pathologic repair of the injured vessel. The disruption of endothelial integrity leads to an associated reduction in the production of vasculoprotective mediators and increased vasoconstrictor and growth-promoting substances, resulting in elevated vascular tone, platelet adhesion, enhanced inflammation, and medial VSMC proliferation\textsuperscript{17}. The resulting neointimal hyperplasia is the basis for restenosis after revascularization procedures such as angioplasty and stenting\textsuperscript{13}.

Vascular endothelial cells produce NO, a vasodilator and the metabolic product of L-arginine by NOS, which plays a critical role in the regulation of vascular homeostasis. Because endothelial cell loss plays a key role in the pathogenesis of neointimal hyperplasia after vascular injury, a therapeutic strategy that promotes re-endothelialization of the injured vessels would inhibit lesion formation and thus improve vessel patency.

Epidemiologic studies have demonstrated that the incidence of coronary artery disease in France is markedly lower as compared with other western countries with a fat containing diet. The French Paradox has been attributed to consumption of red wine in France (an inverse correlation between red wine consumption and incidence of cardiovascular disease)\textsuperscript{324}. RSV, a polyphenolic compound found in grape skins and red wine in high concentrations, has been suggested as one of the agents responsible for the French Paradox. Several biological activities of RSV have been reported, including anti-oxidant activity, cancer chemoprevention, anti-CVD and anti-diabetic properties, and most recently lifespan extension. RSV exerts cardiovascular protection by decreasing LDL oxidation, improving HDL cholesterol levels, inhibiting platelet aggregation and/or adhesion, and decreasing VSMC proliferation or hypertrophy and ECM synthesis (reviewed in Bertelli et al\textsuperscript{275}). In particular, RSV has been shown to decrease
neointimal thickening in animal models of arterial injury\textsuperscript{291-293}, however only Gu et al\textsuperscript{292} performed mechanistic studies, thus the mechanism(s) and signaling pathways by which RSV decreases neointimal formation are still incomplete.

As discussed in the Introduction section 1.7, NO provides significant vascular protection and is produced by different isoforms of NOS enzymes. eNOS and nNOS expression is constant and iNOS is induced by cytokines\textsuperscript{144}. eNOS activation diminishes neointimal formation after arterial injury\textsuperscript{152} and as expected, eNOS deficiency results in greater neointimal growth\textsuperscript{104, 195}. NOS activation represents a likely mechanism for RSV to exert its inhibition on neointimal formation, since RSV enhances expression and activity of eNOS in endothelial cells\textsuperscript{338} and EPCs\textsuperscript{292}. However, NO has not been demonstrated to play a direct role in the effect of RSV to decrease neointimal growth \textit{in vivo}.

Therefore, the objective of our study is to determine the effect of RSV on VSMC growth kinetics over the time course of neointimal formation after balloon catheter injury of the carotid artery in rats. Furthermore, since RSV can stimulate NOS and NO can decrease neointimal growth, we investigated the consequences of NOS inhibition on the effects of RSV.

\textbf{5.3 Materials and Methods}

\textbf{5.3.1 Animal Models}

Six groups of male Sprague-Dawley rats weighing 400-450g and fed normal chow were studied: 1) control (C); 2) RSV (4mg/kg/day, s.c.)-treated (R); 3) RSV + NG-nitro-L-arginine methyl ester (L-NAME) (2mg/kg/day, s.c.) (R+L\textsuperscript{2}); 4) RSV + L-NAME (10mg/kg/day, s.c.) (R+L\textsuperscript{10}); 5) control + L-NAME (2mg/kg/day, s.c.) (C+L\textsuperscript{2}); and 6) control + L-NAME (10mg/kg/day, s.c.) (C+L\textsuperscript{10}). Both doses of L-NAME have been demonstrated to inhibit NO production in the rat aorta, based on vessel cGMP assays\textsuperscript{368}, where only the 10mg/kg/day dose of
L-NAME elevated blood pressure\textsuperscript{369}. The animals were maintained as described in the General Methods section 2.1.

5.3.2 Surgical Procedures

Three days after onset of treatment, injury to either the LCCA or the aorta of the rat was induced by means of an inflated balloon catheter\textsuperscript{133}. Refer to the General Methods section 2.2.4/2.2.5 for details regarding surgical procedures.

5.3.3 Vessel and Blood Sample Collection

The rats were sacrificed in either the fed or fasted state at various time points after injury, based on previous studies investigating the kinetics of vascular injury. Blood samples were collected by cardiac puncture under general anesthesia. Thereafter, rats were sacrificed via anesthetic overdose and the carotids were perfusion-fixed for four minutes with 10% buffered formalin at physiologic pressure (110-120mmHg). The carotids were removed, immersed in 10% formalin for >24h, and embedded in paraffin. Refer to General Methods section 2.3.1 for a complete description.

5.3.4 Metabolic Parameters

Blood glucose was monitored throughout the treatment (see General Methods section 2.3). Plasma insulin was determined by radioimmunoassay using kits specific for rat insulin. Refer to General Methods section 2.12 for details.

5.3.5 Histomorphometry/Matrix Staining

Cell migration into the neointima was measured four days after injury and neointimal cells on the lumen surface were immunostained with an antibody against histone H1\textsuperscript{82}. The number of cells on the luminal surface of the vessel was counted. Refer to General Methods section 2.5.1 for complete details. Neointimal cell proliferation was measured at seven days after injury with an anti-BrdU antibody, as previously described\textsuperscript{82} as neointimal cell proliferation.
reaches a maximum one week after vascular injury\textsuperscript{383}. See General Methods section 2.5.2 for details.

Neointimal cell apoptosis (14 days after injury) was measured by terminal dUTP nick-end labeling (TUNEL) as described in General Methods section 2.5.3. Cross- sectional areas were measured on hematoxylin and eosin stained slides at 14 and 28 days after injury. Cross-sections taken 28 days after injury were also stained with Movat’s pentachrome or PSR dye for elastin and collagen respectively. Refer to the General Methods section 2.5.4 for a detailed description of staining and analysis.

5.3.6 Evan’s Blue Staining

Re- endothelialized area was measured at 14 days after balloon injury and visualized with Evan’s Blue dye. Following perfusion, the thoracic aorta was dissected out and opened longitudinally to observe Evan’s Blue uptake macroscopically (endothelial cells do not stain) (for details see General Methods section 2.5.5). Cross- sectional areas were measured on hematoxylin and eosin stained slides from unopened sections of these aortas as described in the General Methods section 2.5.

5.3.7 Vessel Cannulation

Three days before the hyperinsulinemic- euglycemic clamp, rats were anesthetized with isoflurane and indwelling catheters were inserted into the right internal jugular vein for infusion and the LCCA for blood sampling, as described in the General Methods section 2.2.6.

5.3.8 Hyperinsulinemic- Euglycemic Clamp

After an overnight fast, insulin (5mU/kg.min or 20mU/kg.min) and glucose (variable rate) were infused through the jugular vein for two hours to maintain euglycemia while blood samples were taken from the LCCA for plasma glucose measurements. Refer to the General Methods section 2.10 for a detailed description of the clamp procedure.
5.3.9 Blood Pressure Analysis

Systolic blood pressure was measured indirectly from the tail via the cuff technique, using the Visitech BP-2000 Blood Pressure Analysis System (see General Methods section 2.11 for details). Blood pressure was measured before treatment and throughout at 4, 14, and 28 days after arterial injury.

5.3.10 Statistical Analysis

Values are expressed as mean ± SEM. Statistical analysis was performed using analysis of variance (ANOVA) to test for significant differences between groups and the post hoc comparisons were performed with Tukey’s t-test. Significance was accepted at $P<0.05$.

5.4 Results

5.4.1 Metabolic Parameters

Food, fluid, and total caloric intake were unchanged for RSV- treated rats compared to controls at all time points (Table 5.1). There was no overall effect of treatment on weight change although the C+L2 group gained more weight than controls at seven days. Plasma glucose levels taken over the treatment period were the same in RSV- treated rats and control rats, however plasma glucose was decreased in the R+L2 group at 14 days compared to RSV alone and in the R+L2 and C+L2 groups at 28 days compared to control or RSV alone (Table 5.1). Plasma insulin levels taken at sacrifice were similar in all groups (Table 5.1).

5.4.2 Morphometric Measurements

Treatment with RSV for both 14 and 28 days after balloon injury resulted in decreased neointimal formation (Figure 5.1). This effect was completely blocked by co- treatment with the NOS inhibitor L-NAME at both doses, indicating an NO- dependent effect of RSV. By contrast, there was no change in medial area between the groups (14 days: C=0.17±0.01mm², R=0.17±0.01mm², R+L2=0.16±0.01mm², C+L2=0.17±0.02mm²; 28 days: C=0.15±0.01mm²,
R=0.15±0.01mm², R+L2=0.17±0.01mm², R+L10=0.17±0.01mm², C+L2=0.17±0.01mm², C+L10=0.17±0.01mm²). In addition, there were no differences in external elastic lamina perimeter between the groups at 14 (C=3.0±0.1mm, R=2.8±0.2mm, R+L2=3.0±0.1mm, C+L2=3.0±0.2mm) and 28 days (C=2.8±0.1mm, R=2.8±0.2mm, R+L2=3.1±0.2mm, R+L10=3.0±0.1mm, C+L2=3.0±0.1mm, C+L10=2.9±0.1mm), indicating that there is no vessel remodeling.

Neointimal cell proliferation measured at seven days after vessel injury was decreased with RSV treatment (P<0.01) (Figure 5.2). NOS inhibition with L-NAME abolished the effect of RSV to decrease neointimal cell proliferation. There was no significant difference in the percentage of apoptotic cells in the neointima between control and RSV- treated rats at 14 days after vessel injury (Figure 5.3).

To determine whether the inhibitory effects of RSV on intimal area at 28 days was due to an effect on ECM accumulation, vessel cross- sections were stained with Movat’s pentachrome for elastin or PSR for collagen. RSV significantly decreased elastin accumulation by ~30% (P<0.05) and had a slight tendency to decrease collagen accumulation by ~22% (P=0.40) in the intima of the carotid at 28 days compared to control (Figure 5.4). Furthermore, this effect was prevented by co- administration with L-NAME.

To investigate whether RSV decreased neointimal growth by inhibiting migration, cell migration to the neointima was measured four days after vessel injury. RSV markedly inhibited cell migration by 76%, reducing the number of neointimal cells from 555±59cells/mm² in control rats to 131±27cells/mm² (P<0.001) (Figure 5.5). L-NAME did not alter the effect of RSV to decrease migration (R+L2=70±12cells/mm²).

Analysis of rat aorta specimens after Evan’s Blue staining at 14 days showed that RSV treatment did not affect re- endothelialization of the balloon- injured arterial segments compared
to control (Figure 5.6A, B). Despite this, RSV reduced neointimal thickening in the aorta after balloon injury (Figure 5.6C) \( (P<0.05) \), as was also observed in the carotid artery.

Furthermore, no changes were observed in systolic blood pressure with either RSV or low dose L-NAME (2mg/kg/day) treatment over the course of treatment (Figure 5.7). However, as expected high dose L-NAME (10mg/kg/day) alone and in combination with RSV elevated systolic blood pressure during the course of treatment to 28 days after carotid injury \( (P<0.05) \).

### 5.4.3 Insulin Sensitivity Measurements

RSV has insulin-like and insulin-sensitizing effects. For example, RSV stimulates glucose uptake\(^{279}\) in a skeletal muscle cell line and \textit{in vivo} RSV has been shown to protect against high-fat diet induced insulin resistance in mice\(^{282}\). Preliminary data from our laboratory show that RSV abolishes FFA-induced insulin resistance on glucose uptake in muscle and liver. Furthermore we have previously shown that insulin decreases neointimal growth after carotid balloon injury\(^{133}\). Taken together these data suggest that RSV may decrease neointimal growth indirectly through an increase in insulin sensitivity. Therefore we used a hyperinsulinemic-euglycemic clamp to measure insulin sensitivity in overnight fasted rats after the 28 day treatment with RSV. The 20 mU/kg.min and 5 mU/kg.min insulin infusion rates were used to assess maximum and half-maximum glucose disposal (glucose infusion rate) respectively. The glucose infusion rate was unchanged with RSV treatment (Figure 5.8), indicating that RSV did not affect insulin sensitivity.

### 5.5 Discussion

The present study focused on the \textit{in vivo} effect of RSV on the kinetics of neointimal growth in the rat arterial injury model and the possible involvement of NO in RSV action. In our study, RSV had a marked inhibitory effect on injury-induced migration and proliferation of
neointimal cells, leading to decreased neointimal area. Furthermore, NO appears to play a causal role in the protective effect of RSV on vascular growth.

The inhibitory effect of RSV on both proliferation and subsequent neointimal formation appear to be NO-dependent. However, the decrease in cell migration by RSV was unaffected by L-NAME. Another mechanism by which RSV could inhibit cell migration is by inactivating MAPK, one of the major intracellular signaling pathways critical for migration, as RSV is well known to inhibit MAPK activation in many types of cells (reviewed in \cite{384}). However, as the reduction in neointimal growth and cell proliferation by RSV is completely abolished by NOS inhibition, the inhibitory effect of RSV on cell proliferation may be the primary mechanism by which RSV reduces intimal hyperplasia, regardless of the inhibitory effect on cell migration.

In addition to suppression of proliferation, RSV can also induce apoptosis \cite{337,385}, which could result in a decrease in neointimal formation. However, we did not observe any changes in apoptosis with RSV treatment at 14 days.

The ECM, which is primarily composed of elastin and collagen, is a major component of the neointimal layer and influences neointimal thickening. RSV has been shown to inhibit AGE-induced collagen synthesis in VSMCs \cite{386}, so we wished to see if RSV affected matrix deposition in our model. Elastin staining was significantly decreased with RSV treatment compared to control at the 28 day time point, and there was a tendency for RSV to decrease collagen staining, suggesting that a decrease in matrix deposition may contribute to the net reduction in neointimal growth. Furthermore, NO also affects matrix elements. For example, L-arginine, the substrate for NO production, decreased hyaluronan synthase expression which is an enzyme responsible for making hyaluronan (a component of the ECM) \cite{182}. NO has also been shown to inhibit basal type I collagen levels \cite{183} and collagen synthesis \textit{in vitro} \cite{184}. In our study the effect of RSV to decrease elastin and collagen deposition was prevented with NOS inhibition, again supporting a role of NO in mediating RSV’s protective effects on the vasculature.
Re-endothelialization can decrease neointimal growth and RSV has been shown to accelerate re-endothelialization at 10mg/kg/day and have no effect at 50mg/kg/day administered by oral gavage\(^{292}\). Given the ~40% bioavailability of RSV in rats\(^3^{387}\) and the unknown activity of RSV metabolites after oral administration\(^3^{387}\), our effective dose was possibly lower than the effective dose provided by 10mg/kg/day oral gavage. However, this could explain why we did not see effects on re-endothelialization. Since RSV decreases neointimal growth without delaying re-endothelialization or increasing re-endothelialization, depending on dose, RSV treatment could provide some advantage over the anti-mitogenic agents (rapamycin and paclitaxel) currently used which delay re-endothelialization\(^8\).

Insulin resistance may be a pathogenic factor for endothelial dysfunction possibly through impaired eNOS activity and increased oxidative breakdown of NO\(^3^{388}\). Several diabetic animal models have shown that RSV can activate AMPK and improve insulin sensitivity\(^2^{281},^{282}\). Furthermore, we have previously shown subcutaneous insulin treatment to decrease neointimal formation after arterial injury\(^1^{133}\). Therefore, we wished to address the possibility of RSV improving insulin sensitivity in order to decrease neointimal growth. We found no effect on whole body insulin sensitivity as indicated by the glucose infusion rate during a hyperinsulinemic-euglycemic clamp. Nonetheless, as RSV has been shown to improve insulin sensitivity\(^2^{281},^{282}\) in models of insulin resistance and its mechanism of action is Akt-independent, it is likely that the inhibitory effect of RSV on neointimal formation would remain in an insulin resistant state. This would provide an advantage over insulin as insulin’s inhibitory effect on neointimal growth was shown to be prevented by high-fat feeding\(^1^{133}\).

Hypertension is among the cluster of disorders present in the metabolic syndrome\(^3^{353}\) and available studies have demonstrated that RSV may improve blood pressure in models of hypertension\(^3^{312}\). However, RSV (10mg/kg/day; gavage) treatment alone for up to six weeks did not have an effect on systolic blood pressure\(^3^{312}\), which is in agreement with our study. As
previously mentioned, L-NAME, at specific doses, blocks NO-mediated vasodilatation. However, we purposely used a dose (2mg/kg/day) which was previously shown not to affect blood pressure\textsuperscript{369}. Although not measured in this study, both the 2mg/kg/day and 10mg/kg/day dose of L-NAME have been demonstrated to inhibit NOS based on vessel cGMP assays\textsuperscript{368}. In addition, high dose L-NAME (10mg/kg/day) alone or in combination with RSV, increased systolic blood pressure, as expected, and also prevented RSV’s inhibitory effect on neointimal growth.

In our study we did not investigate the specific NOS isoform involved in RSV action as L-NAME is a general NOS inhibitor. In previous \textit{in vitro} studies RSV increased eNOS expression\textsuperscript{301, 312, 385}, and \textit{in vivo} eNOS expression was enhanced with an oral dose of RSV (2.5-10 mg/kg/day)\textsuperscript{292} that should result in comparable effects to our s.c. dose. Therefore, we hypothesize that eNOS is likely the isoform activated by RSV to inhibit neointimal formation. Unfortunately, the currently available NOS inhibitors do not have specificity for eNOS.

The signaling pathway upstream of NOS by which RSV decreases neointimal formation is not clear. RSV activates AMPK\textsuperscript{282} and AMPK has been demonstrated to phosphorylate and activate eNOS in endothelial cells\textsuperscript{306, 389} and cardiac myocytes\textsuperscript{306}. AICAR, a well known AMPK activator, decreases neointimal formation after arterial injury\textsuperscript{295}. Taken together, this suggests a potential role of AMPK activation in our model, possibly upstream of NOS. Although this has recently been disputed\textsuperscript{284}, many studies indicate that RSV is an activator of sirtuins, mainly SIRT1, which are histone/protein deacetylases shown to be involved in lifespan extension\textsuperscript{390}. Our previous studies \textit{in vitro} demonstrate that RSV stimulates SIRT1 to activate AMPK, since SIRT1 inhibition abolished RSV- stimulated AMPK phosphorylation\textsuperscript{279}. There is also evidence suggesting that SIRT1 is upstream of eNOS as SIRT1 has been shown to deacetylate and activate the eNOS enzyme to promote endothelium-dependent vascular relaxation\textsuperscript{391}. Therefore, activation of SIRT1 may help to explain the action of RSV.
In conclusion, we have shown for the first time that the effect of RSV to decrease neointimal formation after arterial injury is NOS-dependent. These results provide evidence for inhibition of the growth response to vessel injury by RSV in vivo and suggest that RSV treatment may offer protection against restenosis.
Table 5.1. Daily food, fluid and total caloric intake, final weight gain, fed plasma glucose and insulin levels over the treatment periods.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>4 days</th>
<th>7 days</th>
<th>14 days</th>
<th>28 days</th>
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<tbody>
<tr>
<td></td>
<td>C (n=6)</td>
<td>R (n=9)</td>
<td>R+L2 (n=9)</td>
<td>C+L2 (n=7)</td>
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<td>C (n=6)</td>
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<tr>
<td></td>
<td>C (n=9)</td>
<td>R (n=10)</td>
<td>R+L2 (n=10)</td>
<td>C+L2 (n=9)</td>
</tr>
<tr>
<td>Food Intake (g/day)</td>
<td>27±1</td>
<td>25±1</td>
<td>24±1</td>
<td>22±1</td>
</tr>
<tr>
<td>Fluid Intake (g/day)</td>
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<td>32±2</td>
<td>28±1</td>
<td>28±1</td>
</tr>
<tr>
<td>Total Caloric Intake (kCal/g/day)</td>
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<td>83±3</td>
<td>79±4</td>
<td>73±3</td>
</tr>
<tr>
<td>Weight Change (g)</td>
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<td>9±5</td>
<td>6±6</td>
<td>-3±3</td>
</tr>
<tr>
<td>Fed Plasma Glucose (mM)†</td>
<td>6.2±0.1</td>
<td>6.5±0.1</td>
<td>6.1±0.1</td>
<td>6.3±0.2</td>
</tr>
<tr>
<td>Fed Plasma Insulin (pM)¹</td>
<td>89±9</td>
<td>132±10</td>
<td>99±12</td>
<td>146±32</td>
</tr>
</tbody>
</table>

C=Control; R=Resveratrol-treated; L2=L-NAME (2mg/kg/day)-treated. Data are expressed as means ± SEM. **P<0.01, *P<0.05 vs. C; ###P<0.01, #P<0.05 vs. R.

†Data are means ± SEM of individual average glucose levels taken over the treatment period.

¹Data are expressed as means ± SEM and represent values taken at sacrifice.
Figure 5.1. The effect of RSV and NOS inhibition on intimal area. (A) Representative photomicrographs of cross-sections taken at 28 days after carotid injury (x400). (B) Cross-sectional areas of the intima of carotid arteries measured at 14 (C: n=7; R: n=7; R+L2: n=7; C+L2: n=7) and 28 (C: n=9; R: n=10; R+L2: n=10; R+L10: n=8; C+L2: n=9; C+L10: n=8) days after carotid injury. Values are means ± SEM. *P<0.05 vs. C, ###P<0.001 vs. R+L2, #P<0.05 vs. R+L10 at 28 days.
Figure 5.2. The effect of RSV and NOS inhibition on cell proliferation. Cell proliferation expressed as % BrdU-labeled cells in the intima at 7 days after carotid injury (C: n=6; R: n=7; R+L2: n=6; C+L2: n=6). Values are means ± SEM. **P<0.01 vs. C, ###P<0.001 vs. R+L2.
Figure 5.3. The effect of RSV on cell apoptosis. Intimal cell apoptosis expressed as % TUNEL positive cells in the intima at 14 days after carotid injury (C: n= 6; R: n=7). No significant differences were found.
Figure 5.4. The effect of RSV and NOS inhibition on elastin and collagen content. Representative images of staining with (A) Movat’s pentachrome to show elastin and (C) PSR to show collagen in the intima at 28 days after carotid injury. Quantitative analysis of cross-sections stained for elastin (B) or collagen (D) (C: n=9; R: n=10; R+L2: n=10; C+L2: n=9) days after carotid injury. Values are means ± SEM. *P<0.05 vs. C, ###P<0.001 vs. R+L2 at 28 days.
Figure 5.5. The effect of RSV and NOS inhibition on cell migration. (A) Representative photomicrographs showing cells on the lumen surface at 4 days after carotid injury (x400). (B) Quantitative analysis of cell migration (C: n=6; R: n=9; R+L2: n=9; C+L2: n=7). Values are means ± SEM. ***P<0.001 vs. C.
Figure 5.6. The effect of RSV on re-endothelialization. (A) Representative images of Evan’s Blue dye staining of aorta at 14 days after vessel injury. (B) Quantification of re-endothelialized area (% of total vessel area). (C) Cross-sectional areas of the intima in the aorta at 14 days after vessel injury (C: n=8; R: n=9). Values are means ± SEM. *P<0.05 vs. C.
Figure 5.7. The effect of RSV and NOS inhibition on systolic blood pressure. Systolic blood pressure at 3 days before and 4, 14, and 28 days after carotid injury (corresponding to day 0, 7, 17, and 31 of treatment respectively) (C: n=10; R: n=10; R+L2: n=10; R+L10: n=8; C+L2: n=8; C+L10: n=9). Values are expressed as mean ± SEM. Values for each treatment group were compared at each time-point. *P<0.05, **P<0.01, ***P<0.001 vs. C, #P<0.05, ##P<0.01 vs. R.
Figure 5.8. The effect of RSV on whole body insulin sensitivity. Whole body insulin sensitivity is indicated by the glucose infusion rate during the last 30 min of the 2h hyperinsulinemic-euglycemic clamp (C: n=5; R: n=6). Values are expressed as mean ± SEM. No significant differences were found.
6

Summary and Conclusions

6.1 Summary of Each Study in the Thesis

In our previous studies\textsuperscript{133, 134}, rats on insulin treatment and oral glucose to avoid hypoglycemia had reduced neointimal growth after arterial injury. However, plasma glucose in the insulin-treated rats was lower than normal\textsuperscript{133} and the effect of oral glucose remained undetermined. In a separate study by another group\textsuperscript{219} oral sucrose was used instead of glucose to maintain euglycemia with insulin treatment, however under these conditions neointimal growth was increased, unlike in our studies. In Study 1 the effects of insulin, where normal plasma glucose levels were maintained, and oral glucose or sucrose were investigated. Insulin treatment reduced neointimal area after injury in rats receiving oral glucose but not in those receiving oral sucrose. Oral glucose, without insulin, had no effect on neointimal formation whereas oral sucrose alone increased neointimal growth. Furthermore, oral sucrose but not oral glucose decreased insulin sensitivity measured with hyperinsulinemic clamps.

Insulin has protective effects on the endothelium by stimulating NO production and eNOS expression\textsuperscript{235, 236} but also has mitogenic effects on VSMCs \textit{in vitro}\textsuperscript{266}. Furthermore, NOS inhibition has been reported to abolish the effect of insulin to inhibit VSMC migration \textit{in vitro}\textsuperscript{272}. In Study 2, we examined whether NOS was a mediator of insulin’s inhibitory effect on neointimal formation. Therefore, we investigated the consequences of NOS inhibition, with the \textit{in vivo} inhibitor L-NAME, on the effects of insulin. Insulin decreased neointimal area, ECM
production, and cell migration. These effects were abolished by the addition of L-NAME. Furthermore, insulin increased re-endothelialization, which was also completely prevented by co-administration of L-NAME. In addition, insulin increased the expression of VSMC differentiation markers, the number of circulating progenitor cells, and tended to increase eNOS protein expression in the vessel.

RSV has many vasculoprotective effects, including decreasing LDL oxidation, increasing HDL cholesterol, inhibiting platelet aggregation and/or adhesion, and suppressing VSMC proliferation and ECM synthesis (reviewed in Bertelli et al\textsuperscript{275}), several of which are NO-dependent (see Introduction section 1.11 for details). Furthermore, RSV has been shown to decrease neointimal formation after arterial injury\textsuperscript{291-293}, however the mechanism of the effect or the signalling molecules involved remain unclear. In Study 3, our objective was to investigate the mechanism of RSV action and specifically address the NO-dependence of RSV’s inhibitory effect on neointimal formation. RSV reduced neointimal growth, ECM production, intimal cell proliferation, and migration after injury. However, RSV did not affect apoptosis or re-endothelialization. The RSV-induced decrease in intimal area, ECM production, and cell proliferation was abolished by co-treatment with L-NAME while the RSV-induced decrease in cell migration was not. Furthermore, no changes in insulin sensitivity or systolic blood pressure were observed with RSV treatment.

6.2 General Summary

By using the carotid balloon injury model of restenosis, the studies in this thesis have demonstrated that insulin (independent of any glycemic effects) and RSV have a protective effect on the vessel against restenosis and that their effects are mediated by NOS.
6.3 General Conclusion

The results of the studies in this thesis indicate that NO plays a role in mediating the protective effect of insulin and RSV on VSMCs and endothelium *in vivo* to decrease neointimal growth. These results suggest that systemic RSV may have clinical potential in the prevention of restenosis. Systemic insulin cannot be given to non-diabetic patients outside of a supervised facility (i.e. hospital) because of the risk of hypoglycemia. Since both insulin and RSV decrease neointimal formation without negatively impacting re-endothelialization, insulin or RSV treatment could provide some advantage over the anti-mitogenic agents (rapamycin and paclitaxel) currently used in drug-eluting stents, which delay re-endothelialization.
7 General Discussion

7.1 Is the Effect of Insulin Protective or Detrimental in a Model of Restenosis?

Although our understanding of the effect of insulin on restenosis and atherosclerosis has improved, it remains a point of controversy whether insulin itself is vasculoprotective. Several different models have been used in rats to simulate the effect of hyperinsulinemia/insulin resistance and examine neointimal formation; the fructose-fed rat\textsuperscript{213}, the obese Zucker rat\textsuperscript{212}, and the ZDF rat\textsuperscript{214}. The general consensus from these studies was that these rats had greater neointimal formation, however it is not clear whether this is due to the elevated level of endogenous insulin or to insulin resistance (insulin resistance is thought to be primary and cause hyperinsulinemia), in these models as indicated by the tendency of blood glucose to increase.

As a result, our laboratory has focused on examining the relatively short-term effect of exogenous insulin, without insulin resistance, as indicated by the need for treatment with oral glucose. An earlier study in our laboratory determined the effect of insulin on vascular growth after arterial injury in rats and found that intimal area was decreased in insulin-treated rats on a normal diet, however the effect of insulin was diminished by high-fat feeding\textsuperscript{133}. These data suggest that the predominant effect of insulin was to decrease neointimal growth and that insulin resistance, not insulin, is detrimental in the pathogenesis of restenosis.

In contrast to our studies, insulin has also been reported to enhance neointimal formation after vascular injury in normal rats\textsuperscript{219}. These rats were given sucrose water to maintain...
normoglycemia instead of glucose water as in our studies. Therefore, in Study 1 we attempted to
determine the reasons for these differences in results. We eliminated any glucose lowering by i.p.
glucose infusion while using a high insulin dose (5U/day) (decreased neointimal growth), and
included a group receiving oral glucose alone, which did not affect neointimal growth. Oral
sucrose is commonly used to induce insulin resistance in rats, which was confirmed in our
study, and not surprisingly, insulin treatment given with oral sucrose was not effective in
decreasing neointimal growth. Thus, we believe that the increase in neointimal growth observed
when insulin was combined with oral sucrose in Foster’s paper was due to sucrose, not to insulin.

In Study 2, and also in a recently published study by our laboratory, we used a lower
dose of insulin, which allowed us to investigate the effect of insulin concentrations that can be
seen also in non-insulin resistant rats after an oral glucose load, while avoiding glucose-
lowering. Similar to the results of Study 1, insulin decreased neointimal area. This would be in
keeping with a vasculoprotective effect of insulin.

However, Pu et al. have shown that insulin increases neointimal formation. Furthermore, in Indolfi et al. insulin treatment via islet transplantation increased neointimal
growth. One of the major differences between our studies and those of Pu’s and Indolfi’s, is
that in the latter studies insulin was given long before arterial injury (3- 4 weeks) whereas in our
studies insulin treatment prior to injury was relatively short-term (2- 3 days). Therefore, a
possible explanation of the discrepancy with our findings is that the prolonged treatment with
insulin may have resulted in some insulin resistance, as sustained high levels of insulin have
been shown to upregulate a negative feedback loop resulting in inhibition of IRS signalling by
mTOR/S6K1. Thus insulin resistance could then block insulin’s vasculoprotective effects.
Furthermore, insulin also activates a growth-factor like pathway mediated by MAPK, which
remains intact in insulin resistant states. It is possible that the down-regulation of the PI3K-
mediated signalling pathways and continued activity of the insulin- stimulated MAPK signalling pathway, may explain the increase in neointimal formation in some studies. In Study 1 and 2 we did not measure insulin sensitivity in our insulin treatment groups because basal plasma insulin levels were significantly elevated (close to those obtained during the clamp), which interferes with the interpretation of the data from the hyperinsulinemic- euglycemic clamp. In future studies the clamp could be performed soon after the insulin implant is removed, which would reduce basal insulin levels to control. It is likely that the insulin treatment induced some insulin resistance in these groups, however fasting plasma glucose levels were similar in our insulin-treated groups to controls despite oral glucose/ sucrose, suggesting a prevalent state of excess rather than deficiency of insulin action.

Taken together, it appears that under conditions of normal insulin sensitivity, insulin treatment decreases neointimal growth, whereas under conditions of insulin resistance (including insulin- induced insulin resistance) neointimal growth is increased. In our two models of insulin resistance (high fat diet and sucrose feeding) insulin given at the time of arterial injury was not detrimental. However, in Study 1 we have observed an effect of insulin to increase neointimal cell proliferation which remained intact under conditions of insulin resistance. Therefore, we cannot exclude that in other models of insulin resistance and under different experimental conditions, this effect may prevail and lead to neointimal hyperplasia.

Nevertheless, the results of Study 1 and 2 support a protective role of insulin in the vasculature, independent of any glucose- lowering effect, as indicated by a reduction in neointimal formation after arterial injury with insulin administration. Furthermore, oral glucose used to maintain normoglycemia did not affect neointimal formation whereas oral sucrose increased it, which may be explained by sucrose- induced insulin resistance.
7.2 The Protective Effects of Insulin or Resveratrol Against Mechanically-Induced Restenosis: How do They Differ?

In Study 2 and Study 3 we investigated the role of NO in the effect of insulin and RSV to decrease neointimal formation, respectively. The results are summarized in Figure 7.1. In these studies, both insulin and RSV inhibited neointimal formation and cell migration. However, only RSV decreased cell proliferation as in a previous study\textsuperscript{134} we found that insulin did not affect proliferation.

Insulin and RSV both inhibit neointimal growth through a NO-dependent mechanism, despite this, the role of NO in their mechanism of action appears to differ. For example, insulin’s inhibitory effect on cell migration is NO-dependent whereas RSV’s is not. In addition to NO, MAPK is also associated with increased VSMC migration. Therefore, it is not surprising that insulin action involves NO, as insulin is generally thought to activate MAPK or at least not inhibit it. In contrast, as RSV is well known to inhibit MAPK activation in many types of cells (reviewed in \textsuperscript{384}), it is likely that RSV could inhibit cell migration by inactivating MAPK.

NF\kappa B is also associated with increased VSMC migration\textsuperscript{73}. Therefore, insulin and/or RSV may also decrease cell migration through an anti-inflammatory effect. Previously, we found that insulin did not decrease plasma cytokines\textsuperscript{134}, however an effect on inflammatory markers at the level of the vessel has yet to be determined.

As previously mentioned, insulin decreases cell migration and neointimal growth but not cell proliferation\textsuperscript{134} in this model. This suggests a significant role of cell migration inhibition by insulin to decrease neointimal formation. However, unlike insulin, RSV had an inhibitory effect on both cell migration and proliferation, and subsequent neointimal formation. Furthermore, RSV’s inhibitory effect on cell migration was NO-independent whereas the reduction in neointimal growth and cell proliferation by RSV is completely abolished by NO inhibition, implying that RSV’s effect on proliferation is more important than migration. Taken together,
this suggests that inhibition of cell migration may be more important for insulin action than for RSV, and that the inhibitory effect of RSV on cell proliferation may be the primary mechanism by which RSV reduces neointimal growth, regardless of the inhibitory effect on cell migration.

Insulin and RSV also have differing effects on re-endothelialization. Insulin stimulated re-endothelialization, whereas RSV did not affect re-endothelialization. However, other authors have shown that RSV accelerates re-endothelialization at a dose higher than that used in Study 3. Thus, RSV at least does not delay re-endothelialization. After angioplasty, re-endothelialization also occurs from circulating precursors, and the fact that insulin increased the circulating precursors in Study 2 and RSV has been reported to increase circulating EPCs, suggests an additional benefit for both. These data taken together suggest that insulin or RSV could provide some advantage over the anti-mitogenic agents currently used in stents, which delay re-endothelialization. As an additional benefit, both insulin and RSV have been reported to have inhibitory effects on platelet aggregation in vivo, which initiates thrombosis. Therefore, it seems that insulin and RSV have potential for experimentation in a drug-eluting stent, which ideally would inhibit restenosis and thrombosis while at the same time protecting the re-endothelialization process.

The upstream signaling molecules activated by insulin and RSV are also likely to be distinct. It is well accepted that stimulation of eNOS and NO production by insulin is PI3K/Akt-dependent. Less is known regarding the signaling molecules upstream of eNOS in RSV action. RSV has been reported to activate AMPK and AMPK has been demonstrated to phosphorylate and activate eNOS. RSV also activates SIRT1 and studies suggest that SIRT1 may be upstream of AMPK and/or eNOS. The difference between insulin and RSV concerning their signaling pathways may be most relevant in a state of reduced insulin sensitivity. In this state, the insulin-stimulated PI3K/Akt signaling pathway (which is generally thought to be responsible for the vasculoprotective effects of insulin) is blocked. As RSV
signalling is likely not to depend on Akt activation, instead probably involving AMPK, RSV could still be effective in states of reduced insulin sensitivity, thereby offering an advantage over insulin in a clinical setting, which will be further addressed in the Future Directions section 9.0.

7.3 The Role of NO in Insulin and RSV Action: What is the Source of NO?

In the rat arterial balloon injury model used in this thesis, there are several types of cells near the site of injury that express NOS and could therefore be potential sources of NO, however the specific NOS isoform expressed differs between cell types. The cell types that primarily express eNOS include endothelial cells (in an injured vessel there are always endothelial cells along the perimeter of the injured area and as the repair response progresses the endothelial cell layer is restored), circulating EPCs, and platelets. In addition, monocyte-derived macrophages express iNOS and VSMCs express both iNOS and nNOS. It is generally accepted that eNOS and iNOS induction after arterial injury is beneficial, although the effect of iNOS may be more variable because it produces the greatest amount of NO and thus can produce superoxide, which is detrimental, when substrates and cofactors for synthesis of NO are lacking.

The primary finding of Study 2 and Study 3, through the use of the in vivo NOS inhibitor L-NAME, is that stimulation of NO production is the mechanism through which insulin and RSV decrease neointimal formation, respectively. Therefore, the question arises as to which NOS isoform is specifically being affected by insulin or RSV.

Insulin stimulates iNOS in VSMCs. VSMCs are one of the most abundant cell types present particularly during the early stages of the repair response and they express iNOS. Thus representing a potential significant source for insulin-stimulated NO production. Inflammatory cells also express significant amounts of iNOS, and insulin exerts anti-inflammatory effects in vitro and in vivo but we found very few cells positive for CD68 (macrophage marker) or CD45 (general hematopoietic cell marker) after carotid injury. This
suggests that inflammatory cells are not a significant source of NO in our study. Furthermore, we were unable to detect iNOS in the carotid artery via Western blot analysis or RT-PCR. However, insulin is also well known to stimulate eNOS\textsuperscript{234} to increase NO production in endothelial cells\textsuperscript{235}. We have demonstrated that insulin increases the number of circulating precursor cells (which could potentially differentiate into endothelial cells) and promotes re-endothelialization. EPCs and endothelial cells are sites of eNOS- dependent NO production. In addition, we have also shown that eNOS protein expression tended to be increased with insulin treatment in the injured vessel. Taken together, these data indicate that eNOS is the isoform most likely involved in insulin action, however we cannot rule out the possibility of iNOS- dependent NO production in the VSMCs.

The effect of RSV on the different NOS isoforms is similar to that of insulin. RSV increases the expression and activity of eNOS in endothelial cells\textsuperscript{300} and EPCs\textsuperscript{292}. RSV decreased iNOS expression in macrophages\textsuperscript{302}, but also enhanced cytokine- induced iNOS expression in rat VSMCs\textsuperscript{303}. Furthermore, increased expression of both eNOS and iNOS in the heart\textsuperscript{301} was observed in mice treated with RSV. According to the data in Gu et al\textsuperscript{292} there may be a stronger case for RSV action to involve eNOS activation. In the same rat arterial balloon injury model used in this thesis, Gu et al\textsuperscript{292} demonstrated that a high dose of RSV increased re-endothelialization and the number of circulating EPCs, both sites of eNOS- mediated NO production. Furthermore, eNOS mRNA and protein expression were enhanced with RSV in the aorta after arterial injury. However, our studies (Study 3) may support a role of iNOS instead of eNOS, as RSV did not increase re-endothelialization at the dose used in our study. To address the specific NOS isoform involved in insulin or RSV action, immunohistochemistry for eNOS or iNOS on carotid artery cross-sections could be performed followed by studies in a mouse arterial injury model using eNOS or iNOS knockout mice.
Alternatively, perhaps insulin- or RSV- stimulated NO production is not limited to activation of a single NOS isoform. It is possible that during the earlier stages of the repair response, before EPC recruitment and re-endothelialization occur, insulin or RSV primarily activate iNOS in VSMCs to increase NO production and then over time switch to eNOS-dependent NO production in endothelial cells. Ultimately, regardless of the source of NO, the main message is that insulin and RSV decrease neointimal formation through a NO-dependent mechanism. Subsequent studies could specifically address the isoform involved and will be discussed in the Limitations of the Studies section 8.0.
Figure 7.1. Summary of the effects of insulin and RSV in the vessel after arterial balloon injury. (A) Insulin (3U/day) decreased neointimal growth, ECM accumulation, and cell migration, and promoted re-endothelialization, all through a NO-dependent mechanism. VSMC differentiation and the number of circulating progenitor cells were increased, however whether these effects are mediated by NO are not yet determined. (B) RSV decreased neointimal growth, ECM accumulation, and cell proliferation through a NO-dependent mechanism. RSV also decreased cell migration, however this effect was NO-independent.
Limitations of the Studies

1. The cell migration assay used in Study 2 and 3 allows us to measure intimal cell migration \textit{in vivo}, however it cannot distinguish cell origin or type\textsuperscript{45}. In a previous study\textsuperscript{134}, we identified very few macrophages or hematopoietic cells in general, in the medial and intimal layers of control rats after arterial injury, which is consistent with studies by other groups\textsuperscript{17}. Therefore, inflammatory cells are not likely to play a significant role in this model. However, we cannot rule out the possibility of inflammatory cells adhering to the luminal surface immediately following arterial injury. Furthermore, in the same study\textsuperscript{134} we also stained for alpha-actin, a VSMC marker, and found a significant amount of positive stain in the media and intima, suggesting that those cells are primarily VSMCs. Currently, there is much debate concerning the origin of VSMCs present in the intima after arterial injury. As previously mentioned, the medial layer was thought to be the primary source of VSMCs, however other possibilities include adventitial fibroblasts migrating from the adventitia to the intima\textsuperscript{19} and also progenitor cells from either the bone marrow\textsuperscript{112} or within the vessel wall itself\textsuperscript{113}. We have previously found that insulin increases the number of circulating progenitor cells\textsuperscript{134}, which may affect neointimal growth.

2. In addition to VSMC migration and proliferation, ECM accumulation plays an important role in neointimal formation. In Study 2 and 3, we measured collagen and elastin content using immunohistochemical staining for picrosirus red (collagen) and Movat’s pentachrome (elastin)
on vessel cross-sections under light microscopy. The accuracy of this method is limited. More sensitive measurements of collagen and elastin content could be performed using biochemical analysis where $^{14}$C-proline is used to label collagen and elastin, and measure their accumulation and turnover rate$^{90}$. Furthermore, additional components of the ECM could also be measured. Similar to collagen and elastin, proteoglycans (i.e. heparan sulphate) are abundant in the late stages of ECM formation and their accumulation in the neointima could be analyzed. Hyaluronic acid and glycoprotein (i.e. fibronectin, tenascin, thrombospondin, and osteopontin) deposition in the neointima, which are part of the earlier response to arterial injury compared to elastin and collagen, could also be examined in future studies. Immunohistochemical staining of carotid artery cross-sections, as used for analysis of collagen and elastin, or Western blot analysis would be effective techniques to look for changes in several of these ECM components.

3. In Study 2 and 3 we did not identify which isoform of the NOS enzyme is involved in insulin or RSV action. Unfortunately, specific eNOS inhibitors are not currently available and prolonged studies with iNOS specific inhibitors are not feasible due to the high cost. However, in the General Discussion section 7.3, evidence from this thesis and other authors was presented in support of eNOS as the most likely candidate involved in insulin and RSV action to inhibit neointimal formation. This point could be addressed using immunohistochemical staining of vessel cross-sections for eNOS or iNOS, however this method is not quantitative and would not establish a causal role for eNOS or iNOS. Thus, an arterial injury model in eNOS or iNOS knockout mice could be used to see if the protective effect of insulin or RSV is abolished. These studies are currently being performed in our laboratory. The knockout mouse model has limitations as both mouse strains exhibit a phenotype that could interfere with our treatment and results. eNOS knockout mice are hypertensive, insulin resistant, and develop greater diet-induced atherosclerosis and increased neointimal formation (this indicates that eNOS is important in restenosis) after cuff injury compared to wild-type mice. In contrast, iNOS
knockout mice are sensitive to infection, more insulin sensitive, and develop less diet-induced atherosclerosis and decreased neointimal formation after cuff injury than wild-type (reviewed in 396). Thus, it may be difficult to observe changes with insulin or RSV treatment related specifically to the knockout.

4. In Study 2 and 3 the effect of insulin and RSV on systolic blood pressure was measured, however carotid blood flow and diameter was not assessed. Neointimal formation after arterial injury is inhibited by increased blood flow and is mediated, at least in part, by NO369. Insulin has been reported to increase carotid blood flow in uninjured carotids in the absence of changes in blood pressure397. Similarly, RSV286 also increased flow-mediated dilation. Therefore, insulin and/ or RSV could increase blood flow through a NOS-dependent mechanism to inhibit neointimal growth. Furthermore, it is also possible that in addition to a direct effect of NOS increasing flow, a secondary effect of flow-induced activation of NOS contributes to the effect of insulin or RSV. In the future blood flow could be measured using ultrasound techniques.

5. The beneficial health effects of RSV in humans have been attributed mainly to moderate red wine consumption over a long period of time. Furthermore, studies in humans use oral RSV supplements. In Study 3, as in the majority of studies investigating the effects of RSV, RSV was not given orally, which continues to be a major criticism of most studies with RSV. We used subcutaneous implants to administer RSV, as this allowed a more constant and accurate determination of RSV dose, and allowed us to avoid the confounding effects of ultraviolet (UV) light exposure to RSV given in food or water, as UV light isomerizes RSV to an inactive form. Furthermore, oral administration of RSV requires a much higher dose as it is readily metabolized in the digestive tract (the bioavailability of RSV in rats is ~40%387) and also the activity of RSV metabolites after oral administration is unknown. Studies could be repeated with RSV given orally to see if it is still effective, however our preliminary data shows that RSV also decreases neointimal formation after arterial injury in mice when given orally in food.
6. The rat arterial injury model is limited when compared with human restenosis. Inflammation and thrombosis are implicated in human restenosis and are much less after vessel injury in the rat\textsuperscript{21}. For example, we found few macrophages or hematopoietic cells via immunohistochemical staining on carotid artery cross-sections after balloon injury\textsuperscript{134}. This model is also limited because in humans there is compensatory enlargement of the vessel to maintain constant lumen diameter, which does not occur in rats\textsuperscript{17}, and our studies were carried out in normal arteries rather than atherosclerotic arteries. Therefore, we must be cautious in generalizing our conclusions from this model to restenosis in humans. The larger rabbit and pig models mimic human restenosis more closely and it would be interesting to investigate the effect of insulin on neointimal formation after balloon injury in those models. However, the rat model of carotid artery injury is the most well characterized and is very useful for studying kinetics and mechanisms of response to arterial injury\textsuperscript{17}. 
Future Directions

1. In these studies the effects of RSV and insulin were examined in normal rats. In a previous study done by our laboratory as well as in the oral sucrose model of Study 1, the effect of insulin to decrease neointimal formation was abolished when the rats were fed a high-fat diet or oral sucrose to induce insulin resistance. Ultimately, it is in this insulin resistant state, where insulin is less effective, that RSV may have the greatest potential for use clinically. Therefore, it is of clinical importance to determine whether RSV is still effective and can even restore the effect of insulin in this model. Recent preliminary data from our laboratory shows that RSV is still effective to decrease neointimal growth after arterial injury when rats were fed a high-fat diet. Furthermore, RSV also improved whole body insulin sensitivity in these rats as assessed by a hyperinsulinemic-euglycemic clamp, suggesting that perhaps also at the level of the vessel, insulin action may be enhanced.

2. In our studies subcutaneous implants were used, however systemic insulin treatment cannot be given to non-diabetic people due to the risk of hypoglycemia. Furthermore, systemic RSV treatment in humans (mainly orally) may not be as effective due to its rapid conversion to various conjugates by the digestive system and concentrated absorption in various organs (stomach, liver, kidney, intestine), which may limit exposure of the vasculature to RSV. Alternatively, a local delivery of insulin at the level of the vessel, perhaps in a drug-eluting stent, may be beneficial. We have preliminary data showing that insulin applied to the carotid
artery, via a pluronic gel releasing system, decreases cell migration and neointimal growth after balloon injury (see Appendix A). These data suggest that perhaps insulin would be effective in a drug-eluting stent to decrease restenosis, which will be investigated by our laboratory in the future in the rabbit arterial injury model. Since insulin absorbed from pluronic gels is transient (<24 hours) but was still effective, insulin may be infused i.v. as an alternative treatment. This method of delivery is not feasible for chronic treatment but could be performed under supervised hospital conditions at the time of percutaneous intervention in humans. Therefore, it would be interesting to determine if a short duration (<18 hours) of insulin i.v. infusion would have protective effects on the vasculature in the rat restenosis model following balloon injury.

3. As previously discussed in the Introduction section 1.9.2 in detail, it is well known that insulin activates PI3K/ Akt resulting in eNOS stimulation, however this has not yet been examined in the rat arterial injury model. To address the signalling pathway upstream of eNOS, a PI3K inhibitor (i.e. LY294002) could be applied locally on the vessel at the time of injury using the pluronic gel system to see if insulin’s effect on neointimal formation is prevented. Additionally, to implicate PI3K upstream of both Akt and eNOS, Western blot analysis could be performed on carotid arteries from LY294002- treated rats for phosphorylated Akt and eNOS. Akt phosphorylation could also be measured in carotid arteries from L-NAME- treated rats to further provide evidence that Akt is upstream of eNOS.

The signaling pathway upstream of eNOS by which RSV decreases neointimal formation is not clear, however, as discussed in the General Discussion section 7.2, studies indicate a possible role for AMPK and/ or SIRT1. To identify whether RSV’s action to decrease neointimal formation in our model is mediated by AMPK or SIRT1, the respective inhibitors, Compound C and nicotinamide, could be used. Compound C cannot be administered systemically due to toxicity, however it could be applied locally at the time of injury using the pluronic gel releasing system. Furthermore, our laboratory has preliminary data showing that nicotinamide prevents the
RSV-induced decrease in neointimal growth, suggesting that RSV’s effect is also SIRT1-dependent. Alternatively, AMPK or SIRT1 knockout mice could be used to see if RSV is still effective to reduce neointimal formation after arterial wire injury.

4. As mentioned previously, atherosclerosis is a chronic inflammatory response of the arterial wall and both insulin and RSV have anti-inflammatory properties. Furthermore, as we have found that insulin and RSV are protective against restenosis, it would be interesting to expand our investigation to a model of atherosclerosis as well. It is important to investigate models of atherosclerosis in addition to models of restenosis, because, although occlusive arterial disease confers a high risk for CVD events, the majority of myocardial infarctions result from atheroma, the major cause of stenosis.

It is debated whether in animal models of atherosclerosis the prevailing effect of insulin is atherogenic or protective, although the recent studies favour a protective effect due to insulin’s action on the endothelium. Oral insulin, which lowered plasma glucose levels, was shown to decrease plaque formation in ApoE knockout mice. However, insulin treatment still needs to be analyzed where normal glucose levels are maintained and insulin is administered parenterally, as in the treatment of diabetes. In addition, the effect of insulin on plaque stability and the NO dependence of insulin’s effect remain to be investigated. We have preliminary data showing that treatment of chow-fed ApoE knockout mice, a commonly used model of atherosclerosis, for 16 weeks with 0.05U/day insulin significantly decreases lesion area in the descending aorta as shown by staining with oil red O. The same effect is also observed in the proximal aorta and aortic sinus, which are sites of earlier lipid deposition. This is not due to insulin-induced glucose-lowering as glycemia was actually increased in this group of insulin-treated mice given 40% oral glucose. Additional experiments should be completed to maintain glycemia at the same level as in the control group to eliminate any glycemic effect of insulin. However, these studies do support a protective role of insulin in the vasculature.
Studies in models of atherosclerosis, like the hypercholesterolemic rabbit\textsuperscript{286}, and the ApoE\textsuperscript{288} and ApoE/ LDLR knockout\textsuperscript{287} mouse, have shown that RSV is protective against atherosclerosis, as plaque formation was reduced (see Introduction section 1.10.2 for details). However, the mechanism of the effect of RSV has not been elucidated, thus it would be interesting to investigate its possible NO- dependence.
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Local Insulin Application on the Carotid Artery Inhibits Neointimal Formation.

Insulin and Neointimal Formation

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ABSTRACT

OBJECTIVE: Anti-mitogenic agents currently used to prevent restenosis in drug-eluting stents (DES) delay re-endothelialization. Delayed re-endothelialization is now considered as the main cause of late stent thrombosis with DES, which emphasizes the need for new treatments. We have shown that systemic insulin treatment decreases neointimal growth and accelerates re-endothelialization after arterial injury in a rat model of restenosis. However, systemic insulin treatment cannot be given to non-diabetic individuals because of the risk of hypoglycemia. Therefore, we herein investigated whether local insulin treatment is also effective in reducing neointimal growth after arterial injury. METHODS and RESULTS: Rats were given local vehicle (V) or local insulin (I) delivered via pluronic gel applied around the carotid artery immediately following balloon injury. Vessels were collected at 4 and 28 days after injury. Plasma glucose and systemic insulin levels were not affected by local insulin treatment. Insulin decreased intimal area at 28 days ($P<0.05$) and also inhibited cell migration at 4 days ($P<0.05$). CONCLUSIONS: These results are the first demonstration that insulin applied locally on the carotid artery is effective to inhibit the repair response to vessel injury. Therefore, local insulin treatment, possibly via insulin-eluting stents, may have therapeutic potential against restenosis.

Keywords: insulin, pluronic gel, migration, neointima, angioplasty
Percutaneous revascularization procedures, such as angioplasty and stenting, are increasingly used to treat atherosclerotic vascular disease, however these procedures are often followed by restenosis. With balloon-expandable bare-metal stent rates of restenosis are approximately 30%. Drug-eluting stents (DES) were designed to release pharmacological agents after insertion to inhibit vascular smooth muscle cell (VSMC) proliferation and thus restenosis. First-generation DES are coated with either rapamycin or paclitaxel, which have reduced the restenosis rate to less than 10% as compared to the bare-metal stents⁴⁻⁵. However, stent thrombosis has emerged as an infrequent but severe complication³⁻⁴. These agents primarily inhibit VSMC migration and proliferation, which represent crucial events in the development of in-stent restenosis⁵. However, both rapamycin and paclitaxel have been shown to decrease migration and proliferation of mature endothelial cells⁶⁻⁷, as well as proliferation, differentiation, and homing of endothelial progenitor cells⁸⁻⁹, and delay re-endothelialization⁵,10⁻¹². Furthermore, rapamycin and paclitaxel induce the expression of tissue factor, a key factor in the initiation of coagulation and thrombus formation¹³,¹⁴. These effects may be responsible for the increased risk of late stent thrombosis with DES, which has emphasized the need for new treatments.

We have found that subcutaneous insulin treatment decreases neointimal formation¹⁵ and VSMC migration, while accelerating re-endothelialization in a rat model of restenosis¹⁶. Thus insulin if effective locally (systemic insulin treatment cannot be given to non-diabetic people due to the risk of hypoglycemia) could provide some advantage over the anti-mitogenic agents currently used in stents because of this effect on re-endothelialization. As endothelial progenitor cells (EPC) are involved in re-endothelialization after angioplasty¹⁷,¹⁸, EPC trapping stents were developed, which affect VSMC via re-endothelialization¹⁹,²⁰. Over these stents insulin-eluting stents could have the advantage of an additional direct effect to inhibit VSMC migration as
suggested by our studies with subcutaneous insulin\textsuperscript{16}, thus potentially being more effective on restenosis. Furthermore, insulin, at physiological concentrations, has been shown to have inhibitory effects on platelet aggregation, which initiates thrombosis, \textit{in vivo}\textsuperscript{21-24}. Therefore, it seems that insulin has potential for experimentation in a DES, which ideally would inhibit restenosis while at the same time promoting re-endothelialization. However, before the development of insulin-eluting stents, the effectiveness of local insulin treatment needs to be addressed. Therefore, the objective of this study is to determine whether local treatment of the injured vessel with insulin is effective in decreasing neointimal growth.

**METHODS**

**Animal Models.** Two groups of male Sprague-Dawley rats (Charles River) fed rat chow (Purina 5001) weighing 400-450 g were studied: vehicle control and insulin (human; Humulin, Lilly)-treated. Insulin was administered via pluronic gel. Under light isoflurane anesthesia, the left common carotid artery was injured with a 2F balloon catheter (Baxter) which was inflated and withdrawn 4 times, followed by application of 0.5ml pluronic gel solution around the vessel. The solution contained insulin (10nM)/vehicle in 30% gel F-127 in saline\textsuperscript{25,26}. This concentration corresponds to approximately 10 times the plasma concentration of insulin in Chan et al\textsuperscript{16}.

Analgesic (Buprenorphine) was given at the end of each surgical procedure. Blood glucose was monitored throughout the treatment using a glucometer (Glucometer Elite, Bayer Inc.). On the first day of treatment fed blood glucose was measured approximately once per hour and after that once per day at the same time of the day (~10a.m.). Fasting blood glucose was measured once per week. The Animal Care Committee of the University of Toronto approved all procedures.

**Blood and Vessel Sample Collection.** The rats were sacrificed at various time points after injury, based on previous studies investigating the kinetics of vascular injury. Blood samples
were collected by cardiac puncture under general anesthesia. Thereafter, rats were sacrificed via anesthetic overdose and the carotids were perfusion-fixed for 4 minutes with 4% buffered paraformaldehyde at physiologic pressure (110 mmHg). The carotids were removed, immersed in 4% paraformaldehyde for >48h, embedded in paraffin, and divided into two blocks by cutting the midsection of the artery. Cross-sections were taken from the midsection.

**Metabolic Parameters.** Plasma insulin was determined by radioimmunoassay (Linco) using kits specific for rat insulin and with 100% cross-reactivity with human insulin (Humulin R; Lilly) (used for treatment). Plasma FFA was measured using a colorimetric kit from Wako. Plasma triglycerides were also measured using colorimetric kits from Boehringer.

**Histomorphometry/Matrix Staining.** To measure cell migration into the intima, carotids were collected 4 days after injury and intimal cells on the lumen surface were immunostained with an antibody against histone H1 (MAB1276; Chemicon) which does not permeate the internal elastic lamina. The entire luminal surface of the vessel was counted with a minimum of 12 fields taken at a magnification of 20X. Each nucleus that was oriented parallel to the long axis of the vessel was counted, whereas medial cells were perpendicular. A blinded coding system was used to avoid experimenter bias. This migration assay takes advantage of the fact that the first VSMCs appear in the intima 3 to 4 days after injury and do not complete a round of replication until 24h later.

Cross-sectional areas were measured on hematoxylin and eosin stained slides at 28 days after injury. Images were analyzed using a computer-assisted morphometric system (Simple PCI, Compix Inc). Intimal area was measured as the area encompassed by the internal elastic lamina minus the lumen area (in this case lumen area was determined by tracing around the inside edge of the vessel and calculating the area inside). Medial area was measured as the area
encompassed by the external elastic lamina minus the area encompassed by the internal elastic lamina (including lumen area). Lumen area was calculated by subtracting the intimal area and the medial area from the total area encompassed by the external elastic lamina (in this case total area was calculated using the external elastic lamina perimeter), assuming circular geometry of the vessel to avoid fixation artifacts.

**Statistics.** Values are mean ± SEM. Means for vehicle *versus* insulin treatment at each time-point were compared by the two-tailed Student’s *t*-test. Calculations were performed using SAS (Statistical Analysis System, Cary, NC).

**RESULTS**

Fluid and food intake, change in body weight, and both fasting and fed plasma glucose levels taken over the treatment period were the same in local insulin-treated rats and vehicle control rats (Table 1). Plasma hormone and metabolite levels were taken at sacrifice and there were no changes in fed plasma insulin, triglyceride, and free fatty acid levels (Table 2).

Treatment with local insulin immediately following balloon injury resulted in decreased intimal area after 28 days (*P*<0.05) (Figures 1A and 1B). By contrast, there was no change in medial area between the groups (V=0.16±0.01mm\(^2\), I=0.14±0.01mm\(^2\)). Furthermore, lumen area was greater in the insulin-treated group at 28 days (*P*<0.05) (Figure 1C). There were no differences in external elastic lamina perimeter between the groups at 28 days (V=2.9±0.2mm, I=2.7±0.1mm), indicating that outward remodeling did not likely contribute to the increase in lumen area with local insulin treatment.

To investigate whether local insulin, as previously seen with systemic insulin, inhibited cell migration, cell migration from the media to the intima was measured 4 days after vessel injury. Local insulin markedly inhibited cell migration by 85%, reducing the number of intimal
VSMCs from 495±98 cells/mm² in vehicle control rats to 74±24 cells/mm² ($P<0.05$) (Figure 2A and 2B).

**DISCUSSION**

The present study focused on the *in vivo* effect of local insulin application on the injured vessel in the rat carotid injury model of restenosis. Insulin had a marked inhibitory effect on injury-induced cell migration, leading to decreased neointimal formation. We observed no changes in plasma insulin or glucose levels. Therefore, our data provide evidence for a protective effect of insulin on vascular growth, in the absence of any systemic effect. The pathogenesis of restenosis after revascularization procedures is characterized by many processes that also occur in atherogenesis, and both diabetes and metabolic syndrome, which are characterized by insulin resistance, are associated not only with atherosclerosis but also with restenosis. However, apart from the risk provided by minimal or overt hyperglycemia, it is unclear whether insulin resistance or hyperinsulinemia provides additional risk.

A few studies have evaluated the effect of systemic insulin on the arterial response to injury. Some studies suggest that insulin treatment aggravates intimal thickening in models of type 1 diabetes. In models of insulin resistance, such as the Zucker Fatty rat, the fructose-fed rat, the pre-diabetic OLETF rat, and the diabetic ZDF rats, neointimal growth after injury in increased, however it is not clear whether this is due to insulin resistance or to hyperinsulinemia. Insulin treatment of non-diabetic rats did not affect DNA synthesis in injured aortas during normoglycemia or hyperglycemia but decreased DNA synthesis in the presence of hypoglycemia; however intimal area was not measured. There are currently few reports on the effect of insulin on intimal thickness after balloon injury in non-diabetic models and their results are opposite. Chronic insulin treatment via islet transplantation or subcutaneous insulin
infusion resulting in glucose-lowering increased neointimal growth\textsuperscript{41}. In contrast, neointimal growth was greater in mice with a defect in insulin signaling (IRS-2 null mice)\textsuperscript{42}, however it is not apparent whether this was due to decreased insulin action on the vessel or to the abnormal metabolic milieu. We have previously found a similar effect with subcutaneous insulin treatment which decreased neointimal formation after balloon angioplasty in rats\textsuperscript{15}. The discrepancy between our studies\textsuperscript{15,16} and other studies in hyperinsulinemic rodents\textsuperscript{31,41} may be explained by some degree of insulin resistance induced by chronic insulin itself or by the oral sucrose given to the insulin-treated rats in one study\textsuperscript{43}. Therefore, a major finding of our current study is that locally applied insulin on the carotid artery itself at the time of injury decreases neointimal area under euglycemic conditions, without any indication of systemic insulinization or insulin resistance.

The rat carotid balloon injury model is currently the best characterized model of restenosis. The time course of neointimal growth has been well established after balloon angioplasty of the carotid artery in the rat\textsuperscript{44}. Medial VSMC proliferation peaks 2 days after angioplasty, followed by a peak in VSMC migration from the media to the intima at 4 days. Vascular VSMCs replicate in the neointima and reach a maximum proliferation rate by 7 days\textsuperscript{44}. Neointimal growth continues through 14 days after vessel injury and peaks and plateaus at 28 days\textsuperscript{44}. We found that insulin markedly inhibited cell migration into the intima, similar to what we have observed in a previous study with subcutaneous insulin treatment\textsuperscript{16}. Several studies have examined the effect of insulin on VSMC migration \textit{in vitro}, and the results are controversial, as insulin at supraphysiological concentrations stimulated VSMC migration\textsuperscript{45} in some studies but not in others\textsuperscript{46}. At physiological concentrations, insulin by itself did not affect migration\textsuperscript{47}, but inhibited the migration of VSMC stimulated by PDGF\textsuperscript{48} and wounding in the
presence of inducible nitric oxide synthase (iNOS) induction\textsuperscript{47}. The latter finding may have pathophysiological implications since iNOS is upregulated in VSMC after balloon angioplasty\textsuperscript{49}. The migration assay measures histone H1 positive nuclei, thus we cannot definitively distinguish migrated VSMC and infiltrating hematopoietic or progenitor cells.

We focused on VSMC migration rather than proliferation because we have previously found no effect of subcutaneous insulin (3 U/day) treatment on VSMC proliferation in the same model of carotid balloon injury\textsuperscript{16} and thus, reduced proliferation was unlikely to account for the effect of local insulin. At a higher dose (5 U/day), we previously found that insulin increases VSMC proliferation in accordance with \textit{in vitro} studies\textsuperscript{50,51}, despite decreasing neointimal growth\textsuperscript{15}. Since the local insulin levels were likely higher in this study than in Chan et al\textsuperscript{16}, it is possible that proliferation is increased and this will have to be determined.

Systemic insulin treatment increased re-endothelialization in an aortic balloon injury model\textsuperscript{52}. Carotid balloon injury is not the best model to assess re-endothelialization, however it will have to be used to assess the effect of local insulin in future studies. In the systemic insulin treatment model, our preliminary data suggest that the effect of insulin to increase re-endothelialization, decrease VSMC migration, and neointimal growth are NO- dependent, as they are abolished by a nitric oxide synthase inhibitor\textsuperscript{52}. Interestingly, a hormone similar to insulin, insulin-like growth factor- 1 (IGF-1), the systemic administration of which modestly increased neointimal growth in one study\textsuperscript{53}, was reported to decrease neointimal growth when applied locally in pluronic gels, perhaps via decreasing VSMC migration\textsuperscript{54}. Recently, IGF-1 was also found to activate eNOS\textsuperscript{55}.

With local treatment in pluronic gel, sustained release is brief\textsuperscript{66} and release is periadventitial. Despite this, the effect of local insulin was marked, which suggests that sustained
release at an endovascular site as in stents may be even more effective, alone or perhaps in combination with commonly used drugs. In particular, a combination with paclitaxel may be promising as insulin may interfere with the inhibitory effect of rapamycin on mTOR and subsequent VSMC proliferation. Larger animal models, such as the iliac artery injury in rabbits and coronary artery injury in pigs, should be studied once a stent is developed because their arteries are more similar to the human coronary artery.

In conclusion, we have shown for the first time that local insulin treatment applied directly on the carotid artery is effective to decreased neointimal thickness and reduce VSMC migration after arterial injury. These results provide evidence for inhibition of the growth response to vessel injury by insulin in vivo and suggest that local insulin treatment, possibly in the form of a drug-coated stent, may offer protection against restenosis.
FIGURE LEGENDS

**Figure 1.** Representative photomicrographs of cross-sections taken at 28 days after carotid injury (x400) (A). Cross-sectional areas of the intima (B) and lumen (C) of carotid arteries measured at 28 (Local Vehicle: n=9; Local Insulin: n=10) days after injury. Values are means ± SEM. *P<0.05 vs. Local Vehicle.

**Figure 2.** Representative photomicrographs showing intimal cell on the lumen surface at 4 days after carotid injury (x 400) (A). Quantitative analysis of cell migration (Local Vehicle: n=3; Local Insulin: n=5) (B). Values are means ± SEM. *P<0.05 vs. Local Vehicle.
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<th>28 days</th>
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<td>Fluid Intake (g/day)</td>
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<td>Fed Plasma Glucose (mM) ‡</td>
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Table 1. Daily fluid and food intake, final weight change, fasting and fed plasma glucose levels over the treatment period. V=Local Vehicle; I=Local Insulin. Data are expressed as means ± SEM. *P<0.05 vs. Local Vehicle. ‡ Data are means ± SEM of individual average glucose levels measured over the treatment period. On the first day of treatment blood glucose was measured approximately once per hour and after that both fasting and fed blood glucose were measured once per day at the same time of the day (~10a.m.).
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<td>130±41</td>
<td>131±17</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>1.8±0.3</td>
<td>1.9±0.4</td>
</tr>
<tr>
<td>Free Fatty Acids (uEq/L)</td>
<td>428±60</td>
<td>388±19</td>
</tr>
</tbody>
</table>

*Table 2.* Fed plasma levels of insulin, triglycerides, and free fatty acids at 4 and 28 days after arterial injury. V=Local Vehicle; I=Local Insulin. Data are expressed as means ± SEM and represent values taken at sacrifice.
Figure 1

A

Local Vehicle

Local Insulin

B

Intimal Area (mm$^2$)

Local Vehicle (n=9)  Local Insulin (n=10)

C

Lumen Area (mm$^2$)

Local Vehicle (n=9)  Local Insulin (n=10)
Figure 2

A

Local Vehicle

Local Insulin

B

Number of cells/mm²

Local Vehicle (n=3)  Local Insulin (n=5)

*