Assessment of Endothelial Function in Humans and the Endothelial-Protective Effects of 3-hydroxy-3-methylglutaryl coenzyme A Reductase Inhibitors

by

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

Department of Pharmacology and Toxicology
University of Toronto

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2012

Abstract

The endothelium plays an essential role in the regulation of vascular homeostasis and a state of endothelial dysfunction, which develops in the presence of cardiovascular risk factors, may contribute to the development and progression of cardiovascular disease. As such, the measurement of endothelial function, beyond being an experimental tool, may serve as an important tool to complement current risk assessment algorithms in the identification of high-risk patients. Flow-mediated dilation (FMD) is a non-invasive measure of peripheral conduit artery endothelial function that holds great promise. Presently, FMD suffers from methodological heterogeneity and a poor understanding of the various biological components involved in eliciting the dilatory response to a given shear stimulus. We compared both traditional and alternative methods of arterial diameter characterization with regards to their repeatability, nitric oxide-dependency, and their sensitivity in distinguishing between normal and dysfunctional endothelial responses. Our findings emphasize the importance of continuous arterial diameter measurement and suggest that the time to peak FMD is not a useful adjunctive measure of the FMD response.
Given that endothelial dysfunction may be of clinical importance, strategies to correct it or prevent it from occurring may be of benefit. The 3-hydroxy-3-methylglutaryl coenzyme A inhibitors are agents that have demonstrated marked cholesterol-independent, endothelial-protective effects. We investigated the ability of rosuvastatin and atorvastatin to protect against endothelial dysfunction associated with ischemia and reperfusion (IR) injury, and chronic nitrate therapy. Using the FMD technique, we demonstrated, for the first time in humans, that acute rosuvastatin administration protects against IR-induced conduit artery endothelial dysfunction.

Additionally, we demonstrated that this effect likely occurred by a cyclooxygenase-2-dependent mechanism, which may provide mechanistic insight into the observed cardio-toxicity with cyclooxygenase-2 inhibitors. In contrast, we observed that this endothelial-protective effect was lost upon sustained rosuvastatin administration, which may have important implications regarding the generation of sustained cardioprotective phenotypes. Finally, we demonstrated that atorvastatin co-administration prevented the development of tolerance and endothelial dysfunction associated with continuous transdermal nitroglycerin therapy in humans, likely through an antioxidant mechanism. Future studies are needed in disease patients to determine whether the concept of nitrate tolerance needs reconsideration in the presence of vascular-protective agents.
Acknowledgments

I owe a world of gratitude to the multitude of people that guided and assisted me through my PhD program:

I must thank my family for their endless love, support, and encouragement. I would not have achieved what I did without you all.

I must also acknowledge my supervisor, Dr. John Parker, for providing me the opportunity to pursue a degree in human mechanistic research and guiding me through every step in my degree. Thank you for showing me the diligence required to plan and implement human physiological studies and for pushing and encouraging me to achieve my best.

To the nursing, technical staff, and fellows of the Cardiovascular Clinical Research Laboratory at Mount Sinai Hospital in Toronto, one of these studies would have even gotten off the ground were it not for your assistance and expertise. Thank you Sue Kelly, Dianne Locke, Rebecca Pipes, Dr. Amar Uxa, Dr. Justin Mariani, Wilson Chan, Peter Picton, Dr. Sean Balmain, Dr. Paul Smith, Anne Schofield, and Thom Benson.

To my PhD advisory committee members: Dr. Paul Dorian, Dr. Gary Newton and Dr. David Riddick, thank you for your guidance and input, for challenging me and teaching me how to defend my research.

I would also like to thank my “unofficial” committee members/co-supervisors, Drs. Tommaso Gori, Susanna Mak, John Floras, and the late Eduardo Azevedo, for their unique insight, constant suggestions and encouragement. Thank you for making my research that much better. In particular, I would like to thank Dr. Tommaso Gori for his intellectual stimulation, his motivation, his enthusiasm, and his friendship.

To my colleagues, Saverio Dragoni, Giuseppe Di Stolfo, George Thomas, Jonathan DiFabio, and JoAnne Arcand. Thank you all for “teaching me the ropes” and allowing me to get involved in laboratory activities.

Lastly, but definitely not least, I owe a special and sincere thank you to Mary Clare Luca, my sidekick. Thank you for being there through the highs and lows of this endeavour. I can honestly say that the studies described in this thesis would never have been completed without your endless help, support, advice, and encouragement. I share this accomplishment with you.
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<th>Description</th>
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<tbody>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase Akt/protein kinase B</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BH₄</td>
<td>Tetrahydrobiopterin</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>EDHFs</td>
<td>Endothelium-derived hyperpolarizing factors</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>FBF</td>
<td>Forearm blood flow</td>
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<td>FMD</td>
<td>Flow-mediated dilation</td>
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<tr>
<td>FMD₆₀</td>
<td>FMD calculated using the diameter at 60 seconds after cuff deflation relative to the preceding baseline diameter</td>
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<tr>
<td>FMDₘₐₓ-ₙₒₓ</td>
<td>FMD calculated using continuous arterial diameter measurement after cuff deflation relative to the preceding baseline diameter</td>
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<tr>
<td>GTN</td>
<td>Nitroglycerin, glycercyl trinitrate</td>
</tr>
<tr>
<td>HF</td>
<td>Heart failure</td>
</tr>
<tr>
<td>HTN</td>
<td>Hypertension</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-hydroxy-3-methylglutaryl coenzyme A</td>
</tr>
<tr>
<td>ICC</td>
<td>Intraclass correlation coefficient</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<tr>
<td>IPC</td>
<td>Ischemic preconditioning</td>
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<td>IR</td>
<td>Ischemia and reperfusion</td>
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<td>IS-5-MN</td>
<td>Isosorbide mononitrate</td>
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<td>LDL</td>
<td>Low-density lipoprotein</td>
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<tr>
<td>L-NMMA</td>
<td>Levo-N-monomethyl arginine</td>
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<td>mALDH-2</td>
<td>Mitochondrial aldehyde dehydrogenase-2</td>
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<tr>
<td>mK&lt;sub&gt;ATP&lt;/sub&gt;</td>
<td>Mitochondrial ATP-dependent potassium channel</td>
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<tr>
<td>mPTP</td>
<td>Mitochondrial permeability transition pore</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate (reduced)</td>
</tr>
<tr>
<td>Acronym</td>
<td>Term</td>
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<tr>
<td>---------</td>
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</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>PGI$_2$</td>
<td>Prostaglandin I$_2$, prostacyclin</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog deleted on chromosome ten</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>SBP</td>
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Chapter 1  Introduction

1.1.  Endothelial function and dysfunction

Blood is transported throughout the body through the systemic circulation, a series of vessels composed of adventitia, vascular smooth muscle and lined by a single layer of cells known as the vascular endothelium. As blood exits the left ventricle of the heart, it enters the aorta, which branches into narrower muscular arteries as the blood moves further downstream. These large, muscular arteries continue to branch, ending in terminal arterioles within the various peripheral tissues. The arterioles further branch and form the capillary network, which provides nutrients to the tissues and clears waste products. The capillaries eventually coalesce and form venules, which further converge, giving rise to veins. The larger veins ultimately form the superior and inferior vena cava that return the blood into the right atrium of the heart.

Arteries that are classified as conduit or conductance arteries, as their name infers, act to transport blood to the periphery and under normal physiological conditions play a minimal role in pressure and blood flow regulation. The smaller arteries and arterioles, which branch from the larger conduit vessels, act to distribute blood within the organ they supply. Collectively termed the resistance vessels, these small arteries and arterioles act specifically to maintain pressure within a narrow range and represent the primary vessels that are involved in the regulation of arterial blood pressure as well organ perfusion pressure. From the small arteries to the distal arterioles, a considerable drop in pressure occurs, with approximately 50-70% of the pressure drop along the vasculature occurring within these vessels. This pressure drop that occurs in the resistance vessels is critical for proper nutrient exchange to occur within the capillaries.

The endothelium lines the lumen of both arteries and veins and is now recognized to be critical to the regulation of vascular homeostasis and organ perfusion. Specifically, the endothelium is a large paracrine organ that secretes numerous factors regulating vascular tone, cell growth, interactions with platelets and leukocytes, and thrombogenicity. The endothelium senses and responds to numerous internal and external stimuli, both physical and chemical, through complex cell membrane receptors and signal transduction mechanisms, leading to the synthesis and release of various vasoactive, thromboregulatory and growth factor substances.
1.1.1. **Endothelial function**

Traditionally, the vascular endothelium was considered an inert component of the blood vessel wall. However, extensive research in the past 30 years has emphasized the functional importance of the endothelium in regulating normal vascular homeostasis. The suggestion that the endothelium was more than a simple, semi-permeable barrier lining the lumen of blood vessels was made as early as the late 19th century (1). It was in 1980 that Furchgott and Zawadski (2) confirmed this hypothesis when they discovered a potent endothelium-dependent vasodilator. Using intact aortic strips, they discovered that acetylcholine (ACh) required the presence of the endothelium in order to induce smooth muscle cell relaxation. It was their seminal observation that revolutionized the field of vascular biology. Seven years after this landmark observation, it was proposed and then demonstrated by Ignarro et al. (3) and Palmer et al. (4) that Furchgott and Zawadski’s endothelium-derived relaxing factor was indeed nitric oxide (NO). These two separate reports showed that NO was indeed synthesized by endothelial cells from the amino acid L-arginine, and that it appeared to activate guanylyl cyclase in vascular smooth muscle cells, leading to their relaxation.

NO is a highly reactive, gaseous free radical generated upon stimulation from physiological factors such as ACh, serotonin (released during platelet aggregation), bradykinin, thrombin, vascular endothelial growth factor (in response to hypoxia), and shear stress (5). NO is synthesized from L-arginine by the nitric oxide synthase (NOS) enzymes, of which there are 3 well characterized isoforms: endothelial NOS (eNOS), neuronal NOS, and inducible NOS (iNOS). The NOS isoforms share approximately 50–60% sequence homology, have similar structure, and are active only when they form part of a NOS dimer (6). Each nitric oxide synthase monomer is comprised of a N-terminal oxygenase domain, which contains a ferric heme complex and binding sites for tetrahydrobiopterin (BH$_4$) and arginine; and a C-terminal reductase domain that that is structurally homologous to cytochrome P450 reductase (7) and contains binding sites for flavin mononucleotide, flavin adenine dinucleotide, and a reduced nicotinamide adenine dinucleotide phosphate (NADPH). Dimerization is essential for enzymatic activity as it is required for proper 3 dimensional structural formation of the catalytic centre, substrate binding site, and the BH$_4$ binding pocket (6). The NOS enzymes catalyze two sequential monooxygenase reactions: the first reaction involves the hydroxylation of L-arginine to N-hydroxy-L-arginine, which remains bound to the enzyme and is subsequently further
oxidized in a second reaction to generate NO and L-citrulline (6). During the synthesis of NO, electrons derived from the NADPH are transferred to the flavins in the reductase domain and must then be transferred to the heme located in the oxygenase domain of the adjacent monomer, a phenomenon termed domain swapping (5). This is necessary for the conversion from a ferric to ferrous heme that allows the binding of oxygen to catalyze the synthesis of NO.

The synthesis of NO by the eNOS protein is dependent upon the interaction of Ca\(^{2+}\) with calmodulin (8). The Ca\(^{2+}\)/calmodulin interaction has been shown to be important as it increases the rate of electron transfer from NADPH to the heme centre (9). Phosphorylation on serine, threonine, and tyrosine residues is another major form of eNOS regulation. One of the better-described sites of regulation by phosphorylation is the amino acid residues serine 1177 in the human eNOS sequence. Serine 1177 phosphorylation appears important for electron flux within the reductase domain, in particular between the flavins (10). Serine 1177 phosphorylation also been shown to increase the Ca\(^{2+}\) sensitivity of the enzyme (5). Several protein kinases can phosphorylate eNOS at Serine 1177 and can participate in eNOS activation following mechanical and/or hormonal stimulation of endothelial cells, including protein kinase Akt/protein kinase B (Akt) (10).

NO is highly membrane permeable and thus, freely diffuses out of endothelial cells to the adjacent vascular smooth muscle cells, acting in a paracrine fashion to bind the heme moiety of the cytosolic soluble guanylate cyclase enzyme (5). The binding to soluble guanylate cyclase activates the enzyme and catalyzes the dephosphorylation of guanosine triphosphate to cyclic guanosine monophosphate (cGMP). cGMP then activates cGMP-dependent protein kinase and cyclic nucleotide-gated ion channels. NO’s vasodilatory activity is mediated through the phosphorylation of various proteins involved in the regulation of intracellular calcium (Ca\(^{2+}\)) levels. These include inositol 1,4,5-trisphosphate receptor-associated cGMP kinase substrate (11); Ca\(^{2+}\)-sensitive potassium channels (12); and the sarcoplasmic reticulum Ca\(^{2+}\)-adenosine triphosphate (ATP)-ase (13). Collectively, these actions decrease intracellular Ca\(^{2+}\) concentrations and prevent the formation of Ca\(^{2+}\)-calmodulin complexes, required for myosin light chain kinase activation. In addition, cGMP-dependent protein kinase also activates myosin light chain phosphatase, leading to desensitization of contractile elements to Ca\(^{2+}\) (14), and by inhibition of RhoA/Rho-kinase-dependent intracellular contractile signaling (15), both resulting in dephosphorylation of myosin light chain and relaxation.
Additionally, NO has been demonstrated to have many actions beyond vascular smooth muscle cell relaxation that are essential to the regulation of vascular homeostasis. NO also acts within the smooth muscle cell to inhibit proliferation, and reduce smooth muscle cell migration (16). Specifically within the endothelium, NO can act in an autocrine fashion to modulate endothelial permeability, inhibit leukocyte adhesion by controlling the expression of vascular and leukocyte adhesion molecules, promote endothelial cell migration, and increase endothelial cell proliferation (16). NO is also able to diffuse into the cytosol of blood-borne platelets, activating platelet soluble guanylate cyclase and increasing production of cGMP. This results in a reduction in platelet activation, as well as inhibiting platelet aggregation and adhesion (16).

More recently, it has been demonstrated that NO also has the ability to form covalent bonds with cysteine-containing thiol groups, known as S-nitrosylation, which alters protein structure and function and results in important posttranslational modifications of cellular signaling components (17). The S-nitrosylation reaction requires oxidized NO derivatives, such as nitrogen trioxide or peroxynitrite and produces a nitrosothiol species. Unlike the cGMP signaling pathway that has a single downstream effector, S-nitrosylation may modify the function of a plethora of cellular proteins and may help to explain the wide-ranging cellular alterations induced by NO. Examples of proteins that can be S-nitrosylated include caspase-3, leading to inhibition of apoptosis; NADPH oxidase, which results in a suppression of ROS production; the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase, which accelerates Ca\(^{2+}\) uptake into the sarcoplasmic reticulum and contributes to arterial relaxation as mentioned above; and cyclooxygenase (COX)-2, leading to an increase in vasoactive prostaglandin production (17,18). Although the S-nitrosylation of several proteins has been described to result in altered function, relatively few S-nitrosylated proteins have been demonstrated under physiological or pathophysiological situations (5). At present, the degree of S-nitrosylation in the cell and what exact role it may play in cell physiology remains poorly understood. It is likely, though, that the role of S-nitrosylation in the regulation of cellular signaling and function is currently largely underestimated.

Importantly, NO is not the only substance to evoke endothelium-dependent vasomotor changes; there are a number of endothelium-derived factors and signals that cause NO-independent hyperpolarization of the underlying vascular smooth muscle. One such factor is prostaglandin-I\(_2\), or prostacyclin (PGI\(_2\)), that is derived from the metabolism of arachidonic acid by cyclooxygenases (19). PGI\(_2\), like NO, is fairly unstable and has a short half-life (16,20). It acts
to relax blood vessels by activating adenylyl cyclase and increasing cyclic adenosine monophosphate production (16), however, in humans its role in the regulation of vasomotor tone is thought to be quite limited (21). Instead PGI$_2$ appears to contribute to other regulatory roles of the endothelium such as the regulation of platelet function. The endothelium also controls vascular smooth muscle cell relaxation through the release of the endothelium-derived hyperpolarizing factors (EDHFs). These are potent vasodilators that activate different families of potassium channels in the subadjacent vascular smooth muscle, transiently hyperpolarizing the cell membrane and resulting in vascular smooth muscle relaxation (22). The precise entity of all the compounds that comprise the EDHFs have not been completely elucidated, and currently includes hydrogen peroxide, carbon monoxide, hydrogen sulfide, arachidonic acid metabolites from the cyclooxygenases, lipoxygenases and cytochrome P450 pathways, and various peptides (possibly C-type natriuretic peptide). Importantly, it is thought that the presence of certain EDHFs may differ between vascular beds (22). Like PGI$_2$, current evidence indicates that the EDHFs play a minor role in the regulation of normal vascular homeostasis. Instead it is believed that the EDHFs likely act to compensate for loss of NO-mediated vasodilator tone, particularly in the microcirculation, and this appears important in situations when NO bioavailability is reduced (23,24).

The endothelium also has the ability to locally produce and release vasoconstrictor peptides, thereby balancing the properties of the aforementioned vasodilators and maintaining vessel tone. In the normally functioning endothelium, the production of vasoactive substances favours vasodilation; however, under pathophysiological conditions, a vasoconstrictor effect may predominate (see below). Endothelial cells synthesize and express angiotensin-converting enzyme on the cell surface, the enzyme responsible for the conversion of angiotensin I to angiotensin II (25,26). Local angiotensin II production by the endothelium can contribute to vascular function by causing potent vasoconstriction, acting directly on smooth muscle to cause contraction, (27,28) and stimulating NADPH oxidase-mediated reactive oxygen species (ROS) production that can act to scavenge NO (29). Further, the angiotensin-converting enzyme can decrease NO synthesis by promoting the degradation and inactivation of bradykinin (27,28). Collectively, such actions elicit a decrease in regional blood flow.

The vasoconstrictor peptides also include the endothelins (endothelin-1, -2, and -3), which are synthesized upon stimulation by various stimuli including angiotensin II, norepinephrine,
oxidized low-density lipoprotein (LDL), hypoxia, low shear stress, and inflammatory cytokines (30). Their production is inhibited by factors such as NO, PGI₂ and high shear stress (30). The endothelins exert their vascular effects through the endothelin receptors, of which three subtypes have been identified: A, B, and C (30). Of these, the endothelin A and B receptors are the best studied. The receptors vary in their specificity for the 3 endothelin peptides, and activate different signaling pathways. The endothelin-A receptor is found abundantly in vascular smooth muscle, whereas the endothelin-B receptor is predominately found on endothelial cells (30). Activation of the endothelin-A receptor stimulates potent vasoconstriction, whereas activation of the endothelial endothelin receptor stimulates NO release and produces a transient vasodilatory response (30). Of the 3 endothelin peptides, endothelin-1 is the best characterized, and is known to produce concentration-dependent contractions that develop slowly but are long lasting (30,31).

Although PGI₂ is the predominant arachidonic acid-derived factor produced by the endothelium, endothelial cells also possess the capability to produce other prostanoids with vasoconstrictor properties (32). These may include prostaglandin H₂, produced by both COX-1 and COX-2, and thromboxane, produced from prostaglandin H₂ by thromboxane synthase. Interestingly, PGI₂ itself may also possess vasoconstrictor properties in situations where the PGI₂ receptor is inactive (32,33). Arachidonic acid metabolism by cytochrome p450 produces another vasoconstrictor, 20-hydroxyeicosatetraenoic acid, which can depolarize the vascular smooth muscle through inhibition of Ca²⁺-activated potassium channels (34). The synthesis of this vasoconstrictor is regulated by NO, which can inhibit its production by cytochrome p450 (34).

In summary, the endothelium regulates vascular homeostasis through synthesis and release of factors that regulate vascular tone and permeability, inflammatory responses, thrombogenicity, as well as angiogenesis. The endothelium acts to maintain a balance between vasodilation and vasoconstriction, between inflammatory and anti-inflammatory responses, inhibition and promotion of the proliferation and migration of smooth muscle cells, and between prevention and stimulation of the adhesion and aggregation of platelets and between thrombogenesis and fibrinolysis. Upsetting this tightly regulated balance results in a state of dysfunction.

1.1.2. Endothelial dysfunction

The term ‘endothelial dysfunction’ is used to denote a switch from a quiescent state to one of endothelial activation. Physical or biochemical injury to the endothelium impairs normal
endothelial function and is characterized by impaired vascular relaxation and an increased sensitivity to vasoconstrictors, enhanced thrombus formation due to an increase in platelet aggregation and inflammation, and accelerated vascular smooth muscle cell proliferation (21,35-37). One fundamental change that occurs to trigger this dysfunction is an excess production of ROS (38). There are several important sources of vascular ROS production that may be activated by exposure to inflammatory cytokines, growth factors, disturbed flow conditions, and in response to stimuli such as angiotensin II (38). These include NADPH oxidase, xanthine oxidase, lipoxygenases, cytochrome P450, and by uncoupling of the mitochondrial respiratory chain (38). Normally, low levels of ROS are maintained and are necessary for vascular function. ROS levels are controlled by antioxidant enzymes such as superoxide dismutases, catalase, glutathione peroxidase, thioredoxins, and peroxiredoxins (39). When the chronic production of ROS exceeds the capacity of cellular enzymatic and nonenzymatic anti-oxidants, coupled with a proinflammatory environment, is when a state of oxidative stress occurs. Oxidative stress plays a role in virtually all of the cellular responses to endothelial injury, discussed below.

A second fundamental change that occurs within the endothelium to trigger dysfunction and activation is a decrease in bioavailable NO due to the uncoupling of NOS (21). When uncoupling occurs, NOS switches its function from a primarily NO-synthesizing enzyme, to a superoxide-producing enzyme, thus exacerbating endothelial oxidative stress levels (40). NOS uncoupling occurs under lower than optimal concentrations of substrate, L-arginine, or cofactors such as BH4, which is readily oxidized in the presence of ROS (40). NOS uncoupling simply means that the transport of electrons to the ferrous-heme-O2 species, generated during the stepwise activation of oxygen by NOS, is no longer coupled to the oxidation of L-arginine, leading to their oxidative decay (5). The enhanced generation of superoxide is likely to result in the formation of peroxynitrite, a potent oxidant capable of oxidizing sulphhydryl groups as well as hydroxylating and nitrating amino acids, such as tyrosine, tryptophan, and guanine (33). Not only does peroxynitrite consume bioavailable NO but may further enhance superoxide production by oxidation of the zinc cluster within NOS and dissociation of the NOS dimer, as well as oxidizing BH4 (5). Further, peroxynitrite inhibits guanylyl cyclase, inactivates the PGI2 synthase by tyrosine nitration, and further enhances oxidative stress by inhibiting superoxide dismutases. Thus, while eNOS normally helps maintain the NO-mediated quiescent state in the endothelium under normal conditions, it can switch to generate ROS under certain circumstances, initiating a vicious circle of ROS production and endothelial activation.
As mentioned above, the combined decrease in vascular NO bioavailability and increase in vascular ROS production not only impairs vasomotor tone, but impairs the nonthrombogenic surface of the endothelial layer, promoting platelet adhesion and aggregation, as well as deposition of platelets on the surface of the endothelium (41,42). Cellular signaling proteins important for the initiation and amplification of the vascular inflammatory response also become active in the absence of NO and an overabundance of ROS. In particular, protein kinase C (PKC) activation is induced both in the presence of superoxide and peroxynitrite (43,44). PKC activation can reduce NO production, by eNOS phosphorylation on threonine 495, and enhance ROS production by stimulation of the NADPH oxidase system (38,40). Importantly, PKC acts to initiate the production of cytokines, interleukins, and tumor necrosis factor-α, and the increased expression of selectins (E- and P-selectin) and adhesion molecules (vascular cell adhesion molecule-1 and intercellular cell adhesion molecule-1) (42). The expression of these inflammatory mediators and membrane receptors facilitates monocyte and neutrophil adhesion to the vascular wall and transmigration into the subendothelial space (37,45). Once in the interstitium, leukocytes produce ROS in large amounts, further increasing oxidative stress, and release various proteases from granules that contribute to extracellular matrix degradation (37,45). The loss of bioavailable NO and the abundance of ROS also impair both the migration and proliferation of endothelial cells, while promoting vascular smooth muscle cell proliferation (39,42).

Clinically, several cardiovascular diseases and associated risk factors have been associated with endothelial dysfunction, including coronary artery disease (CAD) (46-49), heart failure (HF) (50-52), acute myocardial infarction (53), hypercholesterolemia (54-59), hypertension (HTN) (60-63), smoking (64-67), obesity (68), sedentary lifestyle (69), diabetes mellitus (70,71), and pulmonary hypertension (72). Additionally, numerous pathophysiologival processes have been linked to the presence of endothelial dysfunction, including ischemia-reperfusion (IR) injury and nitrate tolerance (53,73-75). Accumulating evidence suggests that endothelial dysfunction contributes to exercise intolerance, impaired myocardial perfusion, left ventricular remodeling, and lowering of the myocardial ischemic threshold in patients with CAD and/or HF (76,77). The presence of endothelial dysfunction also serves as an early marker of atherosclerosis as demonstrated by the observation that fatty streak progression is associated with increasingly impaired vascular relaxation (78) and is believed to underlie many stages in the progression of
atherosclerosis from onset to the lesions that result in CAD (28,79). Importantly, the presence of endothelial dysfunction may not only be associated with cardiovascular disease but may also precede its development. In a study of offspring of hypertensive patients, endothelial dysfunction was observed despite subjects being normotensive at the time of study. Similarly, endothelial dysfunction has been demonstrated in symptom-free children and young adults at high risk for atherosclerosis (80,81). Indeed, endothelial dysfunction has become a hallmark, and possibly a predictor, of cardiovascular disease.

1.1.3. IR injury and associated endothelial dysfunction

IR injury is a phenomenon of potential clinical relevance that is strongly associated with a state of endothelial dysfunction and activation, as mentioned above. IR injury is defined as cellular or tissue injury resulting from the restoration of blood flow to ischemic tissue. In experimental animal models of acute myocardial infarction, it has been demonstrated that IR injury can account for up to 50% of final myocardial infarct size, thus IR injury contributes substantially to determine the extent of tissue injury. In humans, the principal objective in the treatment of patients undergoing an ischemic episode is early and successful restoration of perfusion to the ischemic area, which remains the principal determinant of final infarct size and clinical outcome (82,83). However, despite optimal and timely reperfusion, the death rate after an acute myocardial infarction is near 10%, and the incidence of cardiac failure is almost 25% (84,85). Such incidence rates may, in part, be the result of reperfusion-induced cellular death. IR injury is a multi-faceted disorder and has been shown to cause reperfusion-induced arrhythmias, myocardial stunning (reversible loss of myocardial contractility) and cell death (84,86). The mechanism of IR injury is relatively well understood. During periods of ischemia, anaerobic glycolysis and hydrolysis of high-energy phosphates result in acidification of the intracellular space (87), which is mirrored, in the case of myocardial ischemia, by decreases in pH in blood samples obtained from the coronary sinus. Protons are moved out of the cytoplasm and into the sarcolemma and mitochondrial matrix. The latter results in a disruption of the mitochondrial proton-motive force that normally acts to pump protons into the matrix to create the electrochemical gradient required for ATP synthesis, leading to an impaired production and eventual depletion of the cell’s ATP (88). Further, ischemic conditions also bring about an excess production of hypoxanthine, whose degradation relies on the oxygen-dependent xanthine oxidases. Upon reperfusion, the accumulated hypoxanthine molecules are degraded in the
presence of oxygen, forming large amounts of ROS as a byproduct, such as superoxide ions and hydroxyl radicals (85). At the level of the mitochondria, the re-introduction of oxygen at the time of reperfusion also results in a burst of ROS formation from highly reduced mitochondrial respiratory chain complexes (89). ROS lead to cellular impairment in a number of ways following IR, including membrane lipid peroxidation, enzyme denaturation, intracellular organelle dysfunction, and, indirectly, through the upregulation of cell adhesion molecules (85,90). Similarly, there is an abrupt increase in Ca$^{2+}$ at the time of reperfusion due to ion accumulation during ischemia (84,91). Increases in Ca$^{2+}$ result in cytosolic and mitochondrial Ca$^{2+}$ overload, leading to cellular hypercontracture, mitochondrial swelling, and cell death (91). Along with ROS, Ca$^{2+}$ also facilitates the opening of a large channel in the mitochondrial inner membrane, the mitochondrial permeability transition pore (mPTP). The immediate consequence of mPTP opening is the collapse of mitochondrial membrane potential and further uncoupling of oxidative phosphorylation, leading to ATP depletion (92). Opening of the mPTP also causes osmotic swelling of the mitochondrial membrane, and the exit of ROS and proapoptotic mediators such as cytochrome c (92,93). Thus, the loss of energy substrates caused by ischemia, along with reperfusion-induced ROS and Ca$^{2+}$ excess, lead to a number of processes, which act synergistically to determine cellular dysfunction and death.

1.1.3.1. IR-induced endothelial dysfunction

Endothelial cells have been demonstrated to be highly resistant to ischemic injury when compared to cardiomyocytes (94-97). In contrast, the endothelium is particularly susceptible and highly sensitive to ischemia coupled with reperfusion (53,73,85,98). Indeed, reperfusion appears to be the major mediator of IR-associated endothelial dysfunction, as endothelium-dependent vasomotor responses remain relatively unimpaired in vessels exposed to ischemia alone (99,100). More importantly, these cells may actively participate in the progression of IR injury as endothelial swelling and dysfunction play an important role in limiting the effective reperfusion of ischemic tissue and post-ischemic recovery after removal of the coronary occlusion (101,102). Indeed, in acute myocardial infarction patients treated with thrombolysis, the degree of coronary endothelial dysfunction following reperfusion is significantly correlated with variables indicative of infarct size and necrosis (53). Damaged and dysfunctional endothelium contributes to and exacerbates IR-induced tissue injury through multiple mechanisms. Central to this phenomenon, as in other cases of endothelial dysfunction, is a
reduction in NO bioavailability secondary to the uncoupling of eNOS. A decrease in bioavailable NO results significant impairs endothelium-dependent vasodilation (96,101,103,104). Uncoupling of eNOS has been associated with increases in ROS production from NADPH and xanthine oxidases, as well as a dysfunctional mitochondrial respiratory chain, as mentioned above. IR injury is similarly associated with a massive endothelial inflammatory response, characterized by leukocyte adhesion, infiltration and formation of lesions (86,105). Endothelial cells are particularly susceptible to undergo apoptosis following IR. This is thought to be due to tumour necrosis factor-α-mediated activation of caspases, as well as mitochondrial cytochrome c release following mPTP formation (86). Importantly, it is believed that apoptotic signals emanating from the endothelium influence the fate of subadjacent cardiomyocytes (98). Thus, while being extremely sensitive to IR injury, the endothelium is, at the same time, a major determinant of the capacity of ischemic organs to recover from IR.

1.1.4. Nitrate tolerance and nitrate-induced endothelial dysfunction

1.1.4.1. Pharmacology of the organic nitrates

Nitroglycerin (glyceryl trinitrate; GTN) and other organic nitrates are potent vasodilators and anti-ischemic agents that have been used in the therapy of stable and unstable angina, and chronic HF for over 130 years (106). One of the advantages of this class of agents is its excellent therapeutic profile; organic nitrates are extremely effective anti-ischemic agents that exert their effects rapidly, and are free of serious side effects (107). Organic nitrates induce vasorelaxation by acting at the vascular smooth muscle through a similar signaling mechanism as endogenous NO described above. Following administration, nitrates are biotransformed, a process that is thought to involve denitrification and eventual release of a NO-related species (108). This NO-related species can then activate soluble guanylate cyclase, subsequently elevating tissue cGMP levels, and leading to the activation of cGMP-dependent protein kinase and cyclic nucleotide-gated ion channels that reduce intracellular Ca²⁺ and elicit relaxation, as described for endogenous NO above (109). The potent vasodilatory effect of nitrates on the venous circulation acts to decrease preload, myocardial wall stress and end-diastolic pressure, which combine to decrease myocardial oxygen consumption (107,110). Within the arterial circulation, nitrates have the unique ability to dilate epicardial vessels while having little effect on resistance vessels (111). Such effects lead to improved perfusion of ischemic areas distal to stenotic segments while preventing the development of coronary steal (107,112). Additional benefit of nitrates
may result from an inhibition of platelet aggregation (113).

The metabolic pathway that results in the release of this bioactive metabolite remains incompletely understood. Early work in this area by Needleman suggested that reduced sulfhydryl groups were essential for GTN biotransformation and that an interaction between GTN and reduced sulfhydryl-containing cellular receptors was required for vasorelaxation (114). However, these studies did not address the mechanism of this interaction. More recently, a report by Chen et al. identified mitochondrial aldehyde dehydrogenase-2 (mALDH-2) as an important component of GTN bioactivation, catalyzing the eventual formation of the bioactive NO-related species and the GTN metabolite glycerol-1, 2-dinitrate (115). This hypothesis is supported by observations from mice deficient in mALDH-2 where GTN-dependent relaxation and blood pressure lowering is impaired (115). These animal data have been confirmed in human studies showing that pharmacologic inhibition of ALDH with disulfiram or genetic inhibition of ALDH-2 activity by the East Asian Glu504Lys point mutation is associated with impaired GTN bioactivation and vasodilator potency (116,117). Importantly, mALDH-2 does not appear to account for all GTN bioactivation in vivo, leading to the hypothesis of two available GTN bioactivation pathways (118). While mALDH-2 is believed to account for bioactivation of GTN at therapeutic doses (nanomolar plasma concentrations), a second pathway has been shown to catalyze GTN bioactivation at millimolar concentrations. Unlike the mALDH-2 pathway, this ‘high concentration’ pathway leads to the formation of measureable amounts of NO (119). The enzyme(s) responsible for this secondary pathway remain to be elucidated however reports suggest that cytochrome P450 enzymes located in the endoplasmic reticulum as likely candidates (108). The clinical relevance of the high concentration pathway in the bioactivation of GTN remains unclear. Of note, the ‘high concentration’ pathway is believed to be the primary bioactivation pathway for dinitrate and mononitrate compounds, including isosorbide dinitrate, isosorbide mononitrate (IS-5-MN), and 1,2-glyceryl dinitrate (108).

1.1.4.2. Nitrate tolerance

Despite the effectiveness of the organic nitrates upon acute administration, their clinical efficacy upon prolonged administration is limited due to the rapid loss of their vasodilatory and anti-ischemic effects. The phenomenon of nitrate tolerance was first documented in a case report from 1888, shortly after the initial clinical application of GTN in 1879 (120). However, in spite of the fact that the medical community had known about this phenomenon for almost 100 years,
the systematic evaluation of nitrate tolerance in clinical settings had only begun to be investigated approximately 35-40 years ago. It is now known that when nitrates are continuously administered, their anti-ischemic effect is lost within 24 hours (107,121). In patients with stable angina, a loss of antianginal and anti-ischemic effects, observed as a reduction in exercise capacity or increased frequency of anginal attacks has been demonstrated following continuous GTN administration (121-126). In HF patients, continuous exposure results in an attenuation of the acute hemodynamic effects of GTN (127-130). The loss of these effects is observed with all routes of administration that achieve stable 24-hour plasma concentrations (107,131) and cannot be overcome with dose increases (126). Tolerance is not exclusive to continuous GTN administration and has been observed following long-term isosorbide dinitrate (132-136), and IS-5-MN (137-139) in patients with stable angina and HF. The incidence of tolerance development with continuous nitrate therapy in clinical practice remains to be clearly established. While it is generally believed that almost all patients develop tolerance when exposed to continuous, stable plasma nitrate concentrations, it has been suggested that the effects of a given nitrate in a given patient may differ (140). Quantification of this phenomenon and assessment of the patient characteristics that lead to this heterogeneity, however, have not been performed. It has been hypothesized that some of this heterogeneity may be the result of differences in the presence or degree of tolerance in the venous and arterial circulations (136,141-143).

1.1.4.3. Cross-tolerance and chronic nitrate-induced endothelial dysfunction

Of relevance, chronic nitrate therapy not only causes tolerance, but also impairs the vasodilatory action of other organic nitrates and NO donors, both in animals (144-146) and humans (141,147-149). This phenomenon termed ‘cross-tolerance’ also comprises an impaired vasodilatory action of endogenously produced NO from the endothelium. This state of nitrate-induced endothelial dysfunction has been demonstrated in animals (144,146), as well as in both the coronary (74) and peripheral circulations (75,150) of humans. As discussed above, the development of endothelial dysfunction with nitrate therapy may have important clinical and prognostic implications in patients with cardiovascular disease. As in ‘classical’ endothelial dysfunction, elevated ROS production appears to be central to nitrate-induced endothelial abnormalities (146,151,152). Aortic segments from chronic GTN-exposed animals were found to have superoxide levels twice those of control animals (146). Interestingly, the removal of the endothelium normalized superoxide levels, pointing to its important role in mediating nitrate tolerance (146,153). A
The major source of this superoxide is believed to be NADPH oxidases, as both animal (152,154) and human studies (155,156) of nitrate tolerance have demonstrated an increased activity of these pro-oxidant enzymes. Chronic nitrate exposure is also characterized by eNOS uncoupling and a decrease in vascular NO bioavailability (75,157), as well as an increased vascular production of peroxynitrite (158-160). An increased activation of certain PKC isoforms (PKCα and PKCε) has been demonstrated in nitrate tolerance (161), which may contribute to the reduced NO production from eNOS (162), as well as enhance NADPH oxidase-mediated superoxide production (163,164). Data additionally indicate that endothelial PGI₂ production is also impaired in the setting of chronic GTN therapy due to a peroxynitrite-mediated tyrosine nitration, and subsequent inactivation, of PGI₂ synthase (165). This enzymatic inhibition may also contribute to an enhanced vasoconstrictive response due to the activation of the thromboxane A₂/prostaglandin H₂ receptor of vascular smooth muscle cells by the unmetabolized prostaglandin H₂ (109,165). GTN-induced oxidative and nitrosative stress may also cause the inhibition of sGC, leading to abnormalities in end organ signaling (165,166). In summary, there is now a large amount of evidence suggesting that chronic nitrate therapy impairs endogenous endothelium-dependent responses and function, which is believed to occur through the impairment of multiple components of endothelial cell function.

1.1.4.4. Mechanisms underlying nitrate tolerance and nitrate-induced endothelial dysfunction

The past 40 years have seen many hypotheses proposed to account for the phenomenon of tolerance and its associated complications. These include the sulfhydryl depletion, neurohormonal activation, volume expansion, and end-organ tolerance hypotheses (summarized in table 1.1). The neurohormonal activation and volume expansion that accompany chronic nitrate therapy are collectively classified as pseudotolerance as they comprise counterregulatory mechanisms that occur in response to nitrate therapy. The sulfhydryl depletion and end organ tolerance hypotheses are classified as vascular tolerance, or true tolerance, as they are believed to be the result of changes to intrinsic vascular processes. While none of the hypotheses proposed to date have consistently and adequately been demonstrated to account for tolerance development, a more likely scenario is that nitrate tolerance is a multifactorial phenomenon and that they all contribute to the manifestation of tolerance (140).
Table 1.1. Proposed hypotheses for the development of nitrate tolerance with continuous administration.

<table>
<thead>
<tr>
<th>Hypothesis</th>
<th>Description</th>
<th>Supporting Evidence</th>
<th>Limitations</th>
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<tbody>
<tr>
<td>Sulfhydryl Depletion</td>
<td>Depletion of reduced thiol groups required for GTN biotransformation – reducing bioavailability and efficacy (114,167,168)</td>
<td>Reduced biotransformation by-product associated with tolerance (169-171)</td>
<td>Thiol supplementation studies are inconsistent (176-179)</td>
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<tr>
<td></td>
<td></td>
<td>Thiol donors can prevent tolerance development (129,172-175)</td>
<td>GTN tolerance associated with cross-tolerance to direct NO donors (145,180,181)</td>
</tr>
<tr>
<td>Neurohormonal activation</td>
<td>Counter-regulatory activation of SNS and RAAS cause vasoconstriction and fluid retention, and blunt the vasodilatory action of nitrates (182,183)</td>
<td>Associated with continuous, not intermittent administration (182)</td>
<td>Vessels exposed to GTN in vitro demonstrate tolerance</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inverse relation demonstrated between neurohormonal activation with nitrates and exercise capacity (183)</td>
<td>Time course of neurohormonal activation differs from tolerance development (184,185)</td>
</tr>
<tr>
<td>Volume expansion</td>
<td>An increase in intravascular plasma volume occurs with continuous GTN (178,182)</td>
<td>Decreased hematocrit observed with nitrate therapy. May reverse preload-reducing effect of nitrates (178,182)</td>
<td>Time course of volume expansion differs from that of tolerance development (178,197)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HCTZ and amiloride pretreatment shown to prevent GTN tolerance (196)</td>
<td>Ability of diuretics to prevent tolerance is inconsistent (198)</td>
</tr>
<tr>
<td>End-organ tolerance</td>
<td>Chronic nitrate exposure may impair end-organ signal transduction</td>
<td>Decreased activity of sGC, cGK, PDEs associated with tolerance (119,144,156,199-202)</td>
<td>Vasodilatory responses to some NO donors have been shown to be preserved in the setting of tolerance (203,204)</td>
</tr>
</tbody>
</table>

SNS, sympathetic nervous system; RAAS, renin-angiotensin system; ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker; HCTZ, hydrochlorothiazide; sGC, soluble guanylyl cyclase; cGK, cyclic guanosine monophosphate-dependent protein kinase; PDE, phosphodiesterase.
More recent data have given particular importance to the “oxidative stress concept” as a key component in this process (108) (Figure 1.1). This hypothesis postulates that GTN-induced mitochondrial ROS formation act to inhibit its own bioactivation by the mALDH-2 enzyme and further augment cytosolic ROS production, leading to uncoupling of the eNOS enzyme, a reduction in NO bioavailability, and endothelial dysfunction. The exact mechanism by which GTN induces mitochondrial ROS production remains incompletely understood. It is likely that GTN causes uncoupling of complex I (205) or interacts with complex II and III (206) of the mitochondrial respiratory chain. Other possible mechanisms of GTN-mediated ROS production include a GTN-induced depolarization of mitochondrial membrane potential and mitochondrial swelling (207). It is thought that GTN-induced superoxide acts to inhibit GTN biotransformation on its own, as well as through the formation of peroxynitrite(206). Interestingly, evidence suggests that GTN-induced mitochondrial ROS are transported out of the mitochondria into the cytosol through the formation of the mPTP. Once in the cytosol, these ROS may activate NADPH oxidases through a PKC-dependent process, and simultaneously inhibit the vasoactive enzymes eNOS and PG12 synthase either directly or through generation of peroxynitrite, all of which contribute to GTN-induced endothelial dysfunction (206). Angiotensin II release that accompanies the neurohormonal activation with chronic nitrate therapy is also likely to play a role in augmenting ROS production through the activation of NADPH oxidases (109).

Importantly, inhibition of mPTP with cyclosporine A prevents the development of GTN-induced endothelial dysfunction, in support of the important role of mPTP formation in mediating GTN-induced endothelial abnormalities (206).

The oxidative stress concept has revived the sulfhydryl depletion hypothesis originally proposed by Needleman and Hunter and has led to a new unified hypothesis for nitrate tolerance development (109,208). Evidence suggests that GTN-induced ROS interact with sulfhydryl groups on the mALDH-2 enzyme leading to its inactivation (209).

Importantly, while there is a large amount of evidence supporting oxidative stress concept of tolerance development, the fact that isosorbide dinitrate and IS-5-MN are not bioactivated by mALDH-2 and do not generate mitochondrial ROS indicates that this mALDH-2 inactivation-mitochondrial ROS trigger hypothesis cannot explain tolerance, cross-tolerance, and endothelial dysfunction associated with these nitrates. Additionally, not all studies have supported the concept of ROS-induced abnormalities as a mechanism for tolerance development. A study
GTN undergoes bioactivation by reduced mALDH-2 (mALDH-SH), producing a NO-related bioactive metabolite (NO$_x$) that subsequently induces a vasodilatory response. GTN bioactivation leads to oxidation of mALDH-2 (mALDH-SS). This may occur directly by GTN itself, or indirectly by GTN-triggered mitochondrial ROS formation. It is believed that GTN-induced mitochondrial ROS originate from the direct interaction of GTN with mitochondrial proteins, likely complexes I and III of the respiratory chain. Evidence from animal models of GTN tolerance suggests that GTN-induced mitochondrial ROS are transported outside the mitochondrion following mPTP formation, where they may activate vascular NADPH oxidase through a PKC-dependent process, and inhibit important vascular signaling enzymes such as eNOS, PGI$_2$-synthase (PGI$_2$-S), and soluble guanylate cyclase (sGC). Inhibition of these enzymes contributes to GTN-induced endothelial dysfunction and cross-tolerance to other organic nitrates and NO donors.
conducted using discarded arterial segments from patients undergoing elective coronary artery bypass who were exposed to continuous GTN demonstrated that although superoxide generation was greater in segments from patients receiving GTN, incremental superoxide generation induced by superoxide dismutase inhibition had no effect on GTN responsiveness (171). Further, the role of mALDH-2 inactivation as a mechanism of tolerance development has been questioned (117,210). Of note, while carriers of mALDH-2 polymorphisms and pharmacologic inhibition of mALDH-2 have shown a significant blunting of vasodilator potency by GTN, this enzyme only appears to account for half of the total bioactivation of GTN in humans (117).

1.2. Assessment of endothelial function in humans

Despite several decades of development and refinement, algorithms for the prediction of cardiovascular risk based on "traditional" or "conventional" risk factors fail to predict a substantial proportion of cardiovascular events (211). In light of its potential importance in cardiovascular disease, the ability to measure endothelial dysfunction would be particularly advantageous, as it ideally would provide an independent predictor of risk that could potentially add to current risk assessment. At the least, endothelial function measurement may provide a view into the impact of traditional risk factors on the vascular wall. Such a measure could be used to predict individuals at highest risk of future cardiovascular events or those with unstable atherosclerotic lesions. Additionally, a direct measure of endothelial function may provide a powerful tool to aid in the treatment of patients with pre-existing cardiovascular disease.

There are many methods of assessing conduit and resistance vessel function in humans. Of these, the most widely used techniques are those used to assess endothelial-dependent vasomotion. Coronary conduit vessel responses can be angiographically evaluated by measuring the change in vessel diameter in response to physiological (shear stress induced following the distal administration of adenosine or papaverine) or pharmacological (ACh, substance P, adenosine, and bradykinin) stimuli. Assessment of coronary microvascular responses involves the placement of a Doppler wire into a coronary artery (usually the mid-left anterior descending artery) and measurement of flow velocities. Such measurements can be performed following the infusion of endothelium-dependent (e.g., ACh) and endothelium-independent (e.g., adenosine and papaverine) agents or after physiological stimuli, such as exercise or the cold pressor test. Using such methodology, an impairment in coronary endothelial responses has been documented
in clinical settings such as CAD (46,47,49), HF (52), hypercholesterolemia (54), and hypertension (54). Studies have also demonstrated that measurement of coronary endothelial function may have prognostic importance. Three separate reports have documented an association between blunted ACh-induced endothelial responses or coronary blood flow and cardiovascular events in patients with mild CAD and endothelial dysfunction (212-214). Although the assessment of coronary vessel responses is considered the method with the greatest clinical relevance, it is not ideally suited to large population studies because of its highly invasive nature and expense. Additionally, studies of the coronary circulation are normally limited to patients referred for coronary angiography and are, thus, subject to selection bias. Importantly, studies have demonstrated that patients with impaired endothelial responses in the coronary vasculature show a similar impairment in peripheral vascular beds, leading to the concept of endothelial dysfunction as a systemic process (215). This has permitted the use of techniques to measure endothelial responses in less-invasive vascular beds such as in peripheral limbs.

The measurement of resistance vessel function by venous-occlusion strain gauge plethysmography in peripheral limbs is similar to coronary vascular assessment in that it utilizes the local infusion of pharmacologic agents to stimulate endothelium-dependent dilation. The intra-arterial infusion of stimuli allows the assessment of peripheral vascular function without the confounding effects resulting from the activation of systemic factors. Several studies have demonstrated an impaired ACh-induced vasodilatory response in patients with risk factors and pre-existing cardiovascular disease (50,55-57,60-62,68,70-72). Measurement of forearm resistance vessel function has also been shown to have prognostic significance in patients with mild CAD (216), angiographically-documented acute coronary syndromes (217), hypertensive patients (218), and in a community-based cohort of elderly adults (219). Although this technique has been widely used, the requirement of arterial cannulation for drug infusions makes repeated studies, and those involving large cohorts difficult. Although a correlation between ACh responses in the coronary circulation and in the forearm has been demonstrated (220), resistance vessels in the forearm do not develop atherosclerosis and thus have a different pathophysiology (48). Thus observations made in forearm resistance vessels may not necessarily reflect changes in the conduit coronary arteries that are particularly predisposed to develop disease. A technical description of forearm venous occlusion strain-gauge plethysmography can be found in Appendix 1.
1.2.1. Flow-mediated dilation (FMD)

With these limitations in mind, David Celermajer and colleagues helped to advance the field of endothelial function measurement with the development of the flow-mediated dilation (FMD) technique in 1992 (80). This technique represents a measurement of conduit artery vascular function in a peripheral limb and involves the measurement of arterial diameter before and after an increase in shear stress by reactive hyperemia (221-223). This hyperemic stimulus is commonly induced through inflation of a pneumatic cuff to a suprasystolic pressure. Cuff inflation results in dilation of the vessels located distal to cuff, due to metabolic and myogenic vasodilatory mechanisms (221,224). Upon cuff release, the large drop in resistance due to this distal vasodilation causes a large increase in local blood flow, which increases the shear stimulus on the intimal surface of the vessel wall (221). This shear stimulus is then dynamically mechanotransduced by various components of the endothelial surface, leading to release of endothelial vasoactive mediators (primarily NO) and vasodilation. Such components appear to include Ca^{2+}-activated potassium channels, which hyperpolarize the endothelial cell membrane upon opening, the glycocalyx (network of membrane-bound proteoglycans and glycoproteins, covering the luminal surface of the endothelium that may transduce extracellular signals via the interaction between proteoglycans and associated core proteins, and the cytoskeleton), integrins (family of transmembrane heterodimers that cluster and activate downstream pathways in response to shear stress), platelet-endothelial cell adhesion molecule-1, the endothelial cytoskeleton itself, tyrosine kinase receptors, and the protein caveolin-1 located within caveolae (225). The FMD technique has many advantages over other methods of endothelial function assessment. It is relatively simple, following adequate training, and is inexpensive to perform. Importantly, FMD protocols are usually noninvasive, allowing the possibility to study large cohorts (including children) and to perform repeated measurements in patients. As well, unlike many coronary vasomotion and forearm plethysmography studies, the FMD technique makes use of a physiological stimulus to induce dilation (shear stress). This is discussed in more detail below. Technical aspects of the FMD technique are described in Appendix 2.

1.2.1.1. FMD and coronary function

FMD of coronary vessels is recognized as an important, normal, physiological response for adequate perfusion of cardiac muscle during increased work/demand of the heart (49). As alluded to previously, impaired coronary artery vasodilation in response to exercise or mental
stress, may lower the threshold for the development of myocardial ischemia (226,227), implying that improvements in coronary artery FMD to a sustained shear stress stimulus, such as what is observed during exercise, may attenuate the development of myocardial ischemia. In this regard, there is evidence linking coronary artery FMD to NO (228), and exposure to sustained shear stress results in upregulation of NOS expression and phosphorylation (229). However, it has not been clearly established that all FMD in the coronary vasculature is NO-dependent (230).

Importantly, a modest correlation has been demonstrated between brachial artery FMD and coronary endothelial function assessed by serial intracoronary ACh infusions (215). A limitation of the study is the fact that ACh-mediated responses, instead of coronary FMD responses, were compared to brachial FMD values. Although NO largely mediates responses to both stimuli, they may stimulate dilation by different mechanisms. However, previous studies have demonstrated a close relation between ACh responses and FMD in coronary vessels (46,227). More recently, a close correlation was demonstrated between FMD responses in the brachial artery and coronary vessels, induced by the distal infusion of ATP, in patients with suspected CAD who were referred for coronary angiography (231).

1.2.1.2. Is FMD NO-dependent?

As with coronary FMD, there is controversy regarding the NO-dependency of the experimental FMD technique. The available data in the literature indicate that endothelial-derived NO primarily governs FMD in the brachial or radial artery, but this is highly dependent upon the nature of the elicited shear stimulus. The first study investigating the role of NO in forearm FMD was performed by Joannides et al. (232). In this report, the investigators measured radial artery FMD before and after an intra-arterial infusion of the NOS inhibitor levo-N-monomethyl arginine (L-NMMA). NO synthesis inhibition completely abolished the FMD response and actually converted the response from dilation to a constriction (from 3.6% to -2.8%). This abolition occurred without changes in peak flow, although L-NMMA decreased the duration of hyperemia, likely altering the stimulus for dilation. In a similar study, Mullen et al. (58) similarly observed a complete blunting of FMD after infusion of L-NMMA (~5.3% to 0.7%) with a 5-minute distal occlusion stimulus. In contrast to the study by Joannides et al., L-NMMA had no effect on either the peak or duration of hyperemia. Interestingly, L-NMMA had no effect on FMD after a 15-minute distal cuff occlusion, indicating recruitment of non-NO vasodilators upon longer cuff occlusion durations. Also, gradual and stepwise increases in blood velocity
induced by stimuli such as hand warming resulted in a FMD response that was not L-NMMA sensitive. Doshi et al. (233) subsequently investigated the effect of cuff placement on FMD responses to L-NMMA. FMD was abolished after L-NMMA with distal cuff placement compared to a partial decrease with proximal cuff occlusion. These results suggested that dilation of arteries previously exposed to ischemia (i.e. during inflation of a cuff proximal to the site of measurement) might also be influenced by dilatory factors other than NO. As well, the absence of transmural pressure that occurs during cuff occlusion may also result in a myogenic stimulus that can further complicate the response to an L-NMMA infusion. Seddon et al. (234) aimed to compare the role neuronal and eNOS isoforms on the FMD response in the radial artery through infusion of a selective nNOS inhibitor and L-NMMA (non-selective NOS inhibitor). Only L-NMMA significantly blunted the FMD response (6.5% to 1.0%). The effect of the NOS inhibitors on either the peak or duration of hyperemia was not reported. More recently, a study by Pyke et al. showed no significant changes in FMD with L-NMMA with 5 and 10-minute occlusions (235). While they did not observe any effect of L-NMMA on peak or duration of hyperemia, a significant decrease in radial artery diameter was noted, which is in contrast to the previously reported mentioned above. As such, this may have adversely impacted the interpretation of the FMD following L-NMMA infusion in this study.

1.2.1.3. Role of sympathetic nervous system in FMD responses
At present, the importance of the sympathetic nervous system as a determinant of the FMD response remains controversial. While it has been demonstrated that sympathetic activation does not affect resistance vessel-forearm blood flow responses (FBF) to ACh or reactive hyperemia (236,237), studies investigating the role of sympathetic activation on conduit artery FMD have been mixed. There is evidence that FMD responses are diurnal in nature (i.e. FMD is low in the morning, when sympathetic activation is considered to be high) (238-240). Other studies (241,242), but not all (243) have shown FMD to be blunted during mental stress. Similarly, Hijmering et al. (237) used lower body negative pressure to elicit a sympathetic response in healthy volunteers and subsequently measured FMD. The application of lower-body negative pressure significantly blunted FMD without altering baseline or peak hyperemic blood flow. In contrast, endothelial-independent dilation in response to GTN infusions was not altered. When the investigators intra-arterially infused the alpha-adrenergic blocker phentolamine, baseline FMD was not altered, whereas FMD in response to sympathetic stimulation was normalized.
The investigators concluded that sympathetic activation induced a specific inhibitory effect on shear-mediated NO release during FMD.

Other studies argue against a role of sympathetic activation in FMD. The intra-arterial administration of phenylephrine ($\alpha_1$-adrenergic receptor agonist) has been shown to have no effect on FMD (244). More recently, Dyson et al. (245) tested the effect of sympathetic activation on FMD by 4 different methods: activation of the muscle chemoreflex, which activates a general sympathetic response with increased total peripheral resistance and sympatoexcitation; mental arithmetic; cold pressor test; and lower body negative pressure. Importantly, the investigators modified the durations of cuff occlusions for each method in order to equalize the shear stress stimulus on the endothelium. After controlling for shear stress, FMD values were blunted only in the cold pressor test. Unlike previous studies, lower-body negative pressure and mental arithmetic did not alter FMD responses. The muscle chemoreflex test induced the greatest overall stress and sympathetic activation, and it was the only condition where there was a reduction in baseline diameter. Nonetheless, absolute FMD was the same as control, such that % FMD was greater than in the other models. The authors concluded that collectively, FMD does not appear to be impaired by acute increases in sympathetic activity. When considered with previous studies, the evidence is, thus, questionable as to whether sympathetic activation plays a role in the forearm FMD response.

1.2.1.4. The prognostic significance of FMD

It is now well established that FMD is impaired in the presence of cardiovascular disease (35,51,80,244), and traditional atherosclerotic risk factors (35,46,58,246-250). However, such a relationship has not always been demonstrated (246,251,252), possibly due to a reduced hyperemic shear stimulus (249). As with traditional risk factors of cardiovascular disease, there are now numerous studies that have prospectively assessed the prognostic and predictive value of FMD measurement (Table 1.2). An inexpensive, noninvasive screening method such as FMD that could provide a "barometer" of cardiovascular disease risk would be particularly advantageous, given the current limitations of traditional risk assessment mentioned above (253). Attenuated brachial FMD has been shown to be a predictor of cardiovascular events in patients with established CAD (254-256), HF (217,257), acute coronary syndromes without ST-elevation (258), postmenopausal women with and without hypertension (259,260), and in patients with peripheral arterial disease (261-263). In addition, FMD was shown to predict
**Table 1.2. Studies investigating FMD as a predictor of prognosis in cardiovascular disease patients, patients at high risk for cardiovascular disease, and asymptomatic adults.**

<table>
<thead>
<tr>
<th>Study</th>
<th>Population</th>
<th>Follow-up</th>
<th>Primary Endpoint</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gokce et al.(261)</td>
<td>187 PAD patients to undergo vascular surgery</td>
<td>30 days</td>
<td>CV death, MI, UA, stroke</td>
<td>45 recorded events. FMD independently predicted events</td>
</tr>
<tr>
<td>Modena et al.(259)</td>
<td>400 hypertensive post-menopausal women</td>
<td>67 months</td>
<td>Hospitalization for CV event</td>
<td>47 recorded events. FMD independently predicted event in patients with no improvement in FMD with 6 months of anti-hypertensive therapy</td>
</tr>
<tr>
<td>Gokce et al.(262)</td>
<td>187 PAD patients to undergo vascular surgery</td>
<td>1.2 years</td>
<td>CVD, death, MI, UA, stroke</td>
<td>35 recorded events. FMD independently predicted of events</td>
</tr>
<tr>
<td>Brevetti et al.(263)</td>
<td>131 PAD patients</td>
<td>23 months (mean)</td>
<td>CV death, MI, UA, Stroke, coronary revascularization, TIA, carotid endarterectomy, peripheral revascularization/ critical limb ischemia</td>
<td>39 recorded events. FMD independently predicts events</td>
</tr>
<tr>
<td>Chan et al.(254)</td>
<td>152 CAD patients</td>
<td>34 months (mean)</td>
<td>CV death, MI, UA, stroke, coronary revascularization, TIA, carotid endarterectomy</td>
<td>22 recorded events. FMD independently predicts events at baseline. During follow-up, lowering of FMD predicted events</td>
</tr>
<tr>
<td>Fathi et al.(255)</td>
<td>444 patients at risk of coronary events</td>
<td>24 months (median)</td>
<td>All-cause mortality</td>
<td>119 recorded events. FMD not predictive of mortality, or CV events in low-risk patients. FMD independently predicts CV events in high-risk patients.</td>
</tr>
<tr>
<td>Frick et al.(264)</td>
<td>398 patients undergoing coronary angiography for chest pain</td>
<td>39 months (mean)</td>
<td>CV death, MI, repeat coronary angiography with documented progression of coronary atherosclerosis, coronary revascularization, UA</td>
<td>44 recorded events. FMD not predictive of CV events</td>
</tr>
<tr>
<td>Study</td>
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<td>Follow-up</td>
<td>Primary Endpoint</td>
<td>Result</td>
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<td>44 recorded events. FMD not predictive of CV events</td>
</tr>
<tr>
<td>Fischer et al.(77)</td>
<td>67 HF patients</td>
<td>46 months (median)</td>
<td>CV death, hospitalization for HF (NYHA class IV, pulmonary edema), heart transplantation</td>
<td>24 recorded events. FMD independently predictive of CV events.</td>
</tr>
<tr>
<td>Meyer et al.(257)</td>
<td>75 HF patients</td>
<td>1.5 years</td>
<td>CV death, chronic inotropic support, implantation of a ventricular assist device</td>
<td>27 recorded events. FMD independently predictive of disease progression or CV death</td>
</tr>
<tr>
<td>Karatzis et al.(258)</td>
<td>98 NSTEMI ACS patients</td>
<td>25 months (mean)</td>
<td>CV death, MI, stroke, UA</td>
<td>20 recorded events. FMD independently predictive of CV events.</td>
</tr>
<tr>
<td>Suessenbacher et al.(256)</td>
<td>68 CAD patients</td>
<td>14 months (mean)</td>
<td>CV death, MI, UA, coronary revascularization, repeat angiography with documented CAD progression</td>
<td>10 recorded events. Repeated, but not single (baseline) FMD predictive of CV events.</td>
</tr>
<tr>
<td>Yeboah et al.(265)</td>
<td>2792 asymptomatic elderly adults</td>
<td>5 years</td>
<td>CV death, MI, stroke, HF, claudication, coronary revascularization</td>
<td>674 recorded events. FMD predictive of CV events but does not add to traditional risk assessment</td>
</tr>
<tr>
<td>Rossi et al.(260)</td>
<td>2264 asymptomatic post-menopausal women</td>
<td>45 months (mean)</td>
<td>CV death, MI, stroke, coronary revascularization, TIA</td>
<td>90 recorded events, FMD independently predictive of CV events</td>
</tr>
<tr>
<td>Yeboah et al.(266)</td>
<td>3026 asymptomatic middle-aged adults</td>
<td>5 years</td>
<td>CV death, MI, stroke, angina, coronary revascularization, resuscitated cardiac arrest</td>
<td>198 recorded events, FMD predictive of CV events but not independent</td>
</tr>
<tr>
<td>Shimbo et al.(267)</td>
<td>842 asymptomatic middle-aged adults</td>
<td>3 years</td>
<td>CV death, MI, stroke</td>
<td>30 recorded events. FMD predictive of CV events but not independent</td>
</tr>
</tbody>
</table>
Table 1.2. (Con’t)

<table>
<thead>
<tr>
<th>Study</th>
<th>Population</th>
<th>Follow-up</th>
<th>Primary Endpoint</th>
<th>Result</th>
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</thead>
<tbody>
<tr>
<td>Shechter et al.</td>
<td>435 asymptomatic middle-aged adults</td>
<td>32 months (mean)</td>
<td>All-cause mortality, MI, stroke, angina, coronary revascularization, HF</td>
<td>48 recorded events. FMD independently predictive of CV events</td>
</tr>
<tr>
<td>Anderson et al.</td>
<td>1574 asymptomatic middle-aged adults</td>
<td>7.2 years (mean)</td>
<td>CV death, MI, stroke, angina, coronary revascularization,</td>
<td>111 recorded events. FMD not predictive of CV events.</td>
</tr>
<tr>
<td>Lind et al.</td>
<td>1016 asymptomatic elderly adults</td>
<td>5 years</td>
<td>CV death, MI, stroke</td>
<td>FMD not predictive of CV events</td>
</tr>
</tbody>
</table>

PAD: peripheral arterial disease; CV: cardiovascular; MI: myocardial infarction; UA: unstable angina; TIA: transient ischemic attack; NYHA, New York heart association; NSTEMI: non-ST elevation myocardial infarction; ACS: acute coronary syndromes.
restenosis after PCI (270,271) and complications after vascular surgery (261). FMD measurement may also have predictive value in older (265) and middle-aged adults (266-268) free of clinical cardiovascular disease at baseline. However, FMD was not demonstrated to independently predict cardiovascular events in all cases (255,265-267) and not all studies have demonstrated FMD to be predictive of CV events (219,255,256,264,269). Although many of these negative studies are hampered by small sample sizes and/or small numbers of recorded events, this is not the case for all. Thus, the ability of FMD to independently predict prognosis has not been fully resolved.

1.2.1.5. Characterization of the FMD response

A major limitation of the FMD technique remains the lack of uniform methodological standards for arterial diameter measurement, which may explain the lack of uniformity in results assessing the predictive value of the measurement. Technical components, such as positioning of the cuff (proximal or distal to arterial diameter measurement) and duration of brachial artery occlusion, differ between laboratories. The heterogeneity in study procedures limits its widespread use and makes comparison and reproducibility between laboratories quite difficult.

Another source of inter-laboratory heterogeneity is the method used to characterize the arterial response following cuff deflation. In particular, there is still no standardized approach on how to acquire diameter post-deflation and there is a large variation in the literature regarding the time frame for post-deflation measurement. The traditional approach to FMD calculation expresses the diameter at 60 seconds after cuff deflation relative to the preceding baseline diameter (FMD\textsubscript{60}) (80). This approach was likely taken due to the technical limitations in acquiring arterial images at the time. Nonetheless, this approach of characterizing the dilatory response at a single time point or within a narrow time window is still utilized by many laboratories (223,272). Assuming that the maximal dilatory response does not occur precisely at 60 seconds in all individuals, FMD\textsubscript{60} may underestimate the true FMD response in a number of individuals. Palinkas et al. (273) were one of the first groups to question the measurement of FMD within discreet time points when they demonstrated a temporal heterogeneity in FMD responses measured in a cohort of CAD patients. They found the mean time to reach peak FMD (time to-FMD\textsubscript{max}) was 87±33 seconds with peak FMD responses ranging from 40-190 seconds. This resulted in FMD\textsubscript{60} being lower than the mean peak FMD and that by 60 seconds only 35% of patients reached their maximum FMD response.
More recently, Black et al. performed a study comparing true peak FMD calculated using continuous arterial diameter measurement after cuff deflation (FMD\textsubscript{max-cont}), FMD\textsubscript{60}, and time to-FMD\textsubscript{max} in young, older fit, and older untrained subjects (272). Similar to Palinkas et al., they found a large proportion of FMD\textsubscript{max-cont} diameters that fell outside the time frames commonly used to assess FMD (60s, 50-70s; 70-90s; 0-90s windows), such that FMD\textsubscript{max-cont} was found to be significantly higher than FMD\textsubscript{60} within all 3 groups. Moreover, no differences were found when comparing FMD\textsubscript{60} between groups. In contrast, a significant group effect was found when FMD\textsubscript{max-cont} was compared, suggesting a greater sensitivity of continuous diameter assessment.

Donald et al. subsequently assessed the reproducibility of FMD\textsubscript{max-cont}, FMD\textsubscript{60}, t-FMD\textsubscript{max}, in healthy volunteers, as well as their discriminatory ability when comparing healthy controls to patients with type II diabetes mellitus and hypercholesterolemia (274). They found that while both FMD measures were repeatable, FMD\textsubscript{max-cont} showed better repeatability (coefficient of variation of 10.6% at 1 week for FMD\textsubscript{max-cont} vs. 14.2% for FMD\textsubscript{60}). However, both FMD measures were equally sensitive in detecting differences between healthy subjects and those with risk factors.

The disparity between FMD\textsubscript{max-cont} and FMD\textsubscript{60} also raises important questions regarding the kinetics of the FMD response. It is possible that additional components of the FMD response, namely time to-FMD\textsubscript{max} may provide complimentary and/or additional information to the traditional measurement of FMD. Black et al. observed a significant increase in time to-FMD\textsubscript{max} between young and older healthy volunteers (272), suggesting that the kinetics of FMD may change with age, possibly due to a decreased arterial compliance (275). Chironi et al. (276) compared various components of the FMD response to traditional risk factors, such as Framingham risk score and carotid intima-media thickness, in 50 asymptomatic subjects without known cardiovascular disease (32% hypertension, 38% hypercholesterolemia, 8% diabetes, 14% smokers). Both FMD\textsubscript{max-cont} and time to-FMD\textsubscript{max} were found to be repeatable (coefficient of variation of 7% for FMD\textsubscript{max-cont} and 3.2% for time to-FMD\textsubscript{max}). FMD\textsubscript{max-cont} was found to negatively correlate with Framingham risk score but not with carotid intima-media thickness. The correlation persisted after adjustment for concomitant therapies. In contrast time to-FMD\textsubscript{max} did not correlate with either Framingham risk score or intima-media thickness. Donald et al. (274) assessed the reproducibility and discriminatory ability of time to-FMD\textsubscript{max} in healthy controls and patients with type II diabetes mellitus and hypercholesterolemia. The authors found
no difference in time to FMD$_{\text{max}}$ values between groups and concluded that this variable added little to traditional arterial diameter measurement. Based on current evidence, the usefulness of time to FMD$_{\text{max}}$ as an adjunct to traditional FMD remains controversial.

1.3. **Treatment of endothelial dysfunction – is it reversible?**

Given its potential importance, a significant amount of research has been devoted to the treatment and/or reversal of endothelial dysfunction by cardioprotective lifestyle and pharmacological interventions (35,277). The possibility that treatment strategies, if implemented early, could prevent the development of atherosclerosis in patients at risk, while preserving tissue perfusion and preventing thrombosis in patients with existing cardiovascular disease has been proposed and has made the treatment of endothelial dysfunction an attractive target (35,48,76). While longitudinal studies directly testing this hypothesis have yet to be performed, it is known that many interventions that improve endothelial function experimentally also reduce cardiovascular events. Indeed, interventions such as smoking cessation, exercise, ACE inhibitors and angiotensin receptor blockers, and glycemic control in diabetes mellitus, all have been shown to improve endothelial function in humans and have favourable effects on incidence of cardiovascular events (277-281). Discussion on the treatment of endothelial dysfunction will focus specifically on the settings of IR injury and nitrate tolerance.

1.3.1. **Treatment of IR-induced endothelial dysfunction**

1.3.1.1. **Ischemic preconditioning**

Studies have demonstrated that exposure to brief periods of ischemia prior to a larger ischemic episode, such as an infarction, may reduce myocardial and vascular IR damage. This phenomenon termed ischemic preconditioning (IPC), was first described in a canine model by Murry, Jennings and Reimer in 1986 (282). In this landmark study, the authors found that open-chest dogs exposed to a sequence of four brief ischemic episodes followed by reperfusion (5 minutes of ischemia, 5 minutes of reperfusion) prior to a larger ischemic insult (a 40-min coronary occlusion followed by 4 days of reperfusion) had a significantly smaller infarct size compared to dogs with no preceding exposure to ischemia, an effect found to be independent of differences in coronary collateral blood flow. Since this initial observation, IPC has been found to be effective in multiple animal and organ systems, including humans (283). The protection
afforded by IPC appears to extend beyond the cardiomyocyte, as a marked preservation of endothelium-dependent vasodilation has been observed following IPC in both animal and human models of IR injury (73,102). Clinically, IPC has a number of possible implications: for instance, it is now well accepted that patients who report angina in the 24 hours prior to a myocardial infarction have a better prognosis (284-286). IPC has been found to have a biphasic cardioprotective profile (i.e., after IPC, two windows of protection have been described): the first phase of IPC lasts 3-4 hours following the initial stimulus; the second (termed delayed/late preconditioning) corresponds to a return of the protective effects 24-72 hours after ischemia (287). Because it cannot be predicted when an ischemic event will occur and because of the broader window of protection, the second window has been proposed to be the IPC phase with the greater clinical relevance (287).

1.3.1.2. **Mechanism of IPC**

A number of hypotheses have been generated regarding the pathways leading to the induction of IPC, and, importantly, the proposed mechanisms for the two distinct phases of IPC appear to be completely different. It has been demonstrated that triggering molecules released during IPC (including adenosine, bradykinin, endogenous opiates, ROS, and possibly NO) (288-292) activate complex signal transduction pathways that incorporate protein kinases and a number of pre-existing effector processes, leading to the early phase of preconditioning. In particular, the reperfusion injury salvage kinase pathway, a term used to denote a group of pro-survival protein kinases that include Akt and Erk 1/2, and PKC-ε has been demonstrated to be an important target of triggering molecules (293). Activation of this cardioprotective pathway is believed to mediate cell survival through various mechanisms, including inhibition of apoptosis and excessive autophagy, activation of eNOS, and increased Ca\(^{2+}\) uptake from the sarcoplasmic reticulum (293). In addition, several studies in both animals and humans have demonstrated that a NO-mediated opening of the mitochondrial ATP-dependent potassium (mK\(_{\text{ATP}}\)) channel is essential for the protective effects of early IPC (290,294). Opening of these channels may mediate cardioprotection by various mechanisms. These include (1) depolarization of the mitochondrial membrane potential, which limits Ca\(^{2+}\) entry during reperfusion; (2) slight mitochondrial matrix swelling, that helps to maintain outer and inner mitochondrial membrane contact sites and matrix integrity required for efficient electron transport; and (3) the generation of very low levels of ROS that trigger protection by limiting the production of higher levels of ROS following IR, thus
aiding in the prevention of mitochondrial damage (92, 290, 295). Further, the opening of mK\textsubscript{ATP} channels, increased NO bioavailability, and a reduced intracellular Ca\textsuperscript{2+} concentration act in concert to prevent mPTP opening, thus promoting cell survival.

The release of triggering molecules during early IPC also leads to the activation of a complex signal transduction cascade that includes the PKC-epsilon, the Src/Lck isoforms of tyrosine kinases, Janus-activated kinases 1 and 2. Activation of these signaling cascades stimulates the downstream activation of normally dormant stress-responsive transcription factors, such as NF-\textkappa B, STAT1 and STAT3 (288). Currently this cascade remains incompletely understood and likely involves the contribution of other as yet unidentified protein kinases and transcription factors. Ultimately, this process results in the upregulation of cardioprotective genes and the synthesis of new proteins that mediate the protection afforded by the late phase of preconditioning (287). Several important proteins have been shown to be essential in mediating this response, including aldose reductase (296), antioxidant enzymes (297), and heat stress proteins (288), with particular importance given to iNOS and COX-2 (298, 299). There is now a substantial body of work in animal models of IR injury demonstrating the upregulation of these enzymes in the setting of IPC and the loss of protection when either iNOS or COX-2 are genetically deleted or pharmacologically inhibited (298-305). Upregulation of these protein mediators appear to stimulate the downstream opening of mK\textsubscript{ATP} channels, which have also been implicated as an important end-effector in the late preconditioning cascade (288). It is worth noting that while all of the factors mentioned above appear important in eliciting the late IPC response, the exact interrelationships among them (in particular iNOS, COX-2, and mK\textsubscript{ATP} channels) are still unclear. Further, these lines of evidence have all been developed in animals and evidence for their exact role in preconditioning in humans is still fairly limited.

1.3.1.3. Pharmacologic preconditioning

Of clinical value, it has been shown that certain pharmacologic agents are able to induce a phenotype that is very similar to that observed with IPC, a phenomenon termed ‘pharmacologic’ preconditioning. This phenomenon has been demonstrated in the myocardium, endothelium, and a number of other tissues via mechanisms that appear similar to those seen with IPC. As mentioned above, triggering molecules such as adenosine, bradykinin, and opioids have been shown to be essential for the preconditioning response and the exogenous administration of these compounds or of agents that act to endogenously activate these pathways has been shown to
confer protection comparable to that of IPC (306-312). For instance, by acting on the NO/cGMP axis, sildenafil and GTN have such pharmacologic preconditioning effects, as demonstrated from a number of laboratories (313-317) including our own (318,319). In studies from our group, sildenafil was shown to preserve endothelial function in healthy humans exposed to IR via a mechanism involving the generation of ROS and the opening of mK\textsubscript{ATP} channels (318). We also recently confirmed in humans that a GTN-induced reversible and subcritical opening of the mPTP during the preconditioning phase might prevent the lethal (“high conductance”) opening that occurs during reperfusion, as described above (319,320). However, despite an improved understanding of the pathophysiology of IR injury and a wide range of potential treatments under investigation, preconditioning remains an unexploited possibility in clinical practice.

1.3.2. Treatment of nitrate tolerance and nitrate-induced endothelial dysfunction

Numerous strategies to prevent the development of nitrate tolerance and nitrate-induced endothelial dysfunction have been tested and have been shown to be successful in experimental models. Unfortunately all of these strategies have limitations such that no treatment is currently accepted as a gold standard.

The most widely accepted approach and the primary strategy used clinically to prevent tolerance is the nitrate free interval, or intermittent nitrate exposure (129,321,322). However, the nitrate free interval may be problematic as the patient does not receive any anti-ischemic benefit during the nitrate-free period and it may lead to the development of rebound ischemia (323-325). This rebound phenomenon may be secondary to the development and persistence of endothelial dysfunction during the nitrate free interval as coronary vasoconstrictor responses to ACh are augmented following patch removal in patients receiving transdermal GTN (326).

As mentioned in table 1.2 other treatment strategies have been attempted that strategically target specific components of the nitrate tolerance response. Based on the sulphydryl depletion hypothesis, studies in animal models of tolerance and in humans have reported preserved hemodynamic responses when nitrate therapy is coupled with thiol supplementation by sulphydryl donors N-acetylcysteine (129,173,175,177) and L-methionine (327,328), but these results have been interpreted as an independent potentiation of GTN responses, rather than a reversal or prevention of tolerance (329-333).

Numerous studies have investigated the effects of ACE inhibitors and angiotensin receptor
blockers as a means of counteracting the neurohormonal activation that occurs in the setting of nitrate tolerance. However, evidence supporting a benefit of ACE-inhibitor coadministration in preventing tolerance in both CAD and HF is inconsistent and conflicting (186-192). Importantly, the effect of ACE inhibitors does not seem to depend on the presence of thiol groups as both thiol-containing and non-thiol-containing ACE inhibitors have been demonstrated to have similar efficacy in preventing tolerance (334). Data concerning the effect of angiotensin receptor blockers in the setting of tolerance have also been mixed, with both positive (335,336) and negative reports (195,337). Although it is difficult to explain the conflicting findings in these studies, factors such as drug dose and the use of different functional tests used to assess tolerance may have played a role.

More recent therapeutic investigations to overcome tolerance have focused on strategies designed to modulate ROS and/or NO production. A number of interventions have been tested both in animal and human models, demonstrating the ability to prevent or reverse nitrate tolerance and/or preserve endothelial responsiveness to ACh. Such interventions include the antioxidant compounds hydralazine (338), carvedilol (193,194), vitamin C (150,339-341) and vitamin E (342). Emphasizing the importance of the endothelium in mediating the development of tolerance, it was demonstrated that the coadministration of high dose folic acid prevented the development of GTN tolerance and GTN-induced endothelial dysfunction in the forearm resistance vasculature of healthy volunteers (343). It is hypothesized that folic acid may act as a weak antioxidant as well as to facilitate the recoupling of the eNOS enzyme (343).

1.3.3. 3-hydroxy-3-methylglutaryl coenzyme A inhibitors

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors are potent cholesterol lowering agents and are among the most widely used prescription medications in the treatment of hypercholesterolemia and CAD. In this regard, HMG-CoA reductase inhibitors have been clearly established as effective strategies in the primary and secondary prevention of cardiovascular events (344-351).

The HMG-CoA reductase enzyme is responsible for the deacylation of HMG-CoA to CoA and mevalonate, and is the rate-limiting enzyme in the biosynthesis of cholesterol (352) (Figure 1.2). The HMG-CoA reductase inhibitors potently inhibit this step in the cholesterol biosynthesis pathway by tightly binding to the HMG binding pocket of the enzyme, making it unavailable to
bind substrate HMG-CoA. Of all the HMG-CoA reductase inhibitors, atorvastatin and rosuvastatin have been shown to bind the tightest to the enzyme due to a greater number of bonding interactions (353). Importantly, HMG-CoA reductase inhibitors are able to bind the HMG-CoA reductase enzyme at nanomolar concentrations and are thus very effective at competing with the natural substrate HMG-CoA, which binds the enzyme at micromolar concentrations (354). In addition to inhibiting cholesterol synthesis, HMG-CoA reductase inhibitors contribute to cholesterol reduction by increasing hepatic LDL receptor activity, which occurs in response to reductions in serum cholesterol. These receptors represent the primary mechanism for LDL clearance from the circulation and thus, their upregulation acts to further reduce serum cholesterol levels.

Clinical studies have documented significant reductions in LDL and total cholesterol levels following oral HMG-CoA reductase inhibitor administration. Owing to tighter enzymatic binding, atorvastatin and rosuvastatin have demonstrated to be the most potent of the HMG-CoA reductase inhibitors, achieving between 55-65% reductions in serum LDL at the highest orally administered dose of both agents (355). Similar reductions in triglyceride levels are observed among the HMG-CoA reductase inhibitors with as much as a 40% reduction in serum triglycerides observed in some trials (355). In contrast, currently available HMG-CoA reductase inhibitors appear to have a minor effect on high-density lipoprotein levels, with a 5% average increase observed in clinical studies (355,356).

1.3.3.1. Cholesterol-independent effects of the HMG-CoA reductase inhibitors
It is well established that elevated serum cholesterol is a major risk factor for atherosclerosis and that lowering serum cholesterol is associated with a reduced risk for cardiovascular events (357). Indeed, aggressive lipid lowering is thought to contribute to atheromatous plaque stabilization and modification of atherosclerosis progression (358). This, and the fact that the majority of serum cholesterol is from hepatic origin, has led to the acceptance that the inhibition of cholesterol synthesis is the primary mechanism responsible for the clinical benefits of HMG-CoA reductase inhibitor therapy in cardiovascular disease patients. However, data from some clinical studies have suggested that the efficacy associated with these agents might be the result of effects beyond the reduction in serum lipids (described in section 1.3.3.3).

Subsequent studies have demonstrated important cholesterol-independent effects of the HMG-
CoA reductase inhibitors, such as vasculoprotective and immunomodulatory effects, that may provide a significant additional contribution to the clinical benefit associated with these agents. Through the inhibition of cholesterol synthesis, HMG-CoA reductase inhibitors also inhibit the formation of important byproducts of this pathway, namely isoprenoid intermediates (352,359) (Figure 1.2). The isoprenoids, farnesyl-pyrophosphate and geranylgeranylpyrophosphate, serve as important lipid attachments for the post-translational modification and proper functioning of a number of cellular proteins (352,360). Isoprenoid attachments are required for proper subcellular localization, covalent attachment, and intracellular trafficking of proteins such that their inhibition renders these membrane-associated proteins inactive (352,360). Such proteins comprise approximately 2% of cellular proteins and include small guanosine triphosphate binding proteins, such as Ras and Rho (361). In the absence of isoprenylation, Ras and Rho are unable to bind guanosine triphosphate and remain inactive in the cytosol. With regards to the cholesterol-independent effects of the HMG-CoA reductase inhibitors, particular importance has been given to the Rho family, which includes RhoA, Rac1, and Cdc42. These small guanosine triphosphate binding proteins have been shown to regulate functions actin cytoskeletal changes, microtubule dynamics, vesicle trafficking, cell polarity, and cell-cycle progression (361). These cellular functions, in particular cytoskeletal rearrangements, may also affect the intracellular localization of numerous other proteins and could modify their intracellular transport, membrane trafficking, mRNA stability and gene transcription. Such alterations can have profound effects on the various cells and proteins that regulate functions of the vascular wall.

1.3.3.2. **Effects of the HMG-CoA reductase inhibitors on the endothelium**

There is now a substantial amount of data demonstrating the ability of HMG-CoA reductase inhibitors to improve endothelium-dependent vasodilatory responses in the coronary and peripheral circulations of hypercholesterolemic and CAD patients as well as HF patients (362-369), although negative studies do exist (370-372). One of the major mechanisms responsible for the improvement in endothelial function with the HMG-CoA reductase inhibitors appears to be the stimulated increase in NO bioavailability. Through the inhibition of RhoA, HMG-CoA reductase inhibitors act to stabilize eNOS mRNA, increasing mRNA half-life and therefore eNOS mRNA expression (373). Inhibition of RhoA also appears to increase eNOS enzymatic activity. HMG-CoA reductase inhibition is associated with an activation of the phosphatidylinositol 3-kinase(PI3K)/Akt signaling pathway that can phosphorylate the eNOS
Inhibition of HMG-CoA reductase decreases the downstream production of cholesterol as well as isoprenoid intermediates such as geranylgeranylpyrophosphate. This leads to an inhibition of isoprenylation, and subsequent inactivation, of small GTPases such as Rho, which includes RhoA, Rac1, and Cdc42. Inactivation or Rho GTPases leads to modulation of various cellular functions.

GTPases, guanosine triphosphate-ases.
enzyme and increase its activity (352,374). HMG-CoA reductase inhibitors have also been shown to reduce caveolin-1 expression, which normally competes with calmodulin for binding to eNOS, thus promoting increased calmodulin binding and enzymatic activation (361,375). HMG-CoA reductase inhibitors may also act to recouple uncoupled eNOS by increasing production of BH4 through the upregulation of guanosine triphosphate-cyclohydrolase-1, the rate-limiting enzyme responsible for BH4 synthesis (376,377). Thus, HMG-CoA reductase inhibitors act to increase NO bioavailability through multiple targets, both at the mRNA level and at the protein level. This increase in bioavailable NO associated with HMG-CoA reductase inhibition also appears to decrease platelet reactivity and aggregation, as well as contribute to a decreased inflammatory response (described below).

HMG-CoA reductase inhibitors have also been shown to decrease the action of the endothelial vasoconstrictor peptides. HMG-CoA reductase inhibitors can decrease the synthesis of endothelin-1 and cause a downregulation of both angiotensin type 1 and endothelin-A receptors (361,378-380). HMG-CoA reductase inhibitors also appear to improve endothelial function through antioxidant effects. In particular, HMG-CoA reductase inhibitors have been demonstrated to attenuate NADPH oxidase mediated superoxide production. The GTP binding protein Rac1, an essential component of the NADPH oxidase system, is inhibited following treatment with HMG-CoA reductase inhibitors (352,381,382). Further, HMG-CoA reductase inhibitor treatment indirectly modifies NADPH-mediated superoxide production by downregulating the angiotensin type 1 receptor (352,382), mentioned above. Another indirect antioxidant effect may be the recoupling of NOS mentioned above, thus decreasing NOS-mediated superoxide production (377). HMG-CoA reductase inhibitors may also alter endothelial responses by modulating vascular inflammation. A decreased expression of intercellular adhesion molecule-1 and P-selectin has been observed following HMG-CoA reductase inhibitor administration, which may thus attenuate leukocyte-endothelial interactions (352,383-385). Importantly, there is evidence to suggest that, rather than a direct effect, the decreased expression of P-selectin is the result of an increase in NO bioavailability (385).

1.3.3.3. Evidence for cholesterol-independent effects in clinical trials and mechanistic studies in humans

As mentioned above, it was initially assumed that the clinical benefit associated with HMG-CoA reductase inhibitor administration was solely due to serum cholesterol reduction. Indeed, meta-
analyses of trials involving HMG-CoA reductase inhibitors suggest that lipid modification alone accounts for the associated clinical benefits as a strong relation was noted between percent LDL reduction and percent reduction in cardiovascular events (352,386). However, when compared to trials involving other lipid-lowering strategies such as cholestyramine treatment and partial ileal bypass surgery, trials involving HMG-CoA reductase inhibitor therapy have tended to show greater benefit in a shorter amount of time (5 years vs. greater than 7 years), despite similar reduction in LDL levels (386). Furthermore, in the Heart Protection Study and the Anglo-Scandinavian Cardiac Outcome Trial, the relative risk reduction in patients who received HMG-CoA reductase inhibitor therapy was independent of the baseline lipid levels (349,350). Additional subgroup analysis in patients with similar on-treatment serum cholesterol levels from the West of Scotland Coronary Prevention Study also showed a 36% lower risk of subsequent events, respectively, in patients administered HMG-CoA reductase inhibitors compared to placebo (345,346). More recently, the “Justification for the Use of statins in Prevention: an Intervention Trial Evaluating Rosuvastatin” trial assessed the effect of rosuvastatin on cardiovascular events in an apparently healthy older study population with normal LDL levels and elevated serum high-sensitivity C-reactive protein levels, a marker of systemic inflammation (387). Results from this trial revealed that rosuvastatin significantly reduced the incidence of major cardiovascular events and further subanalysis showed that asymptomatic individuals randomized to rosuvastatin benefited particularly if low concentrations of both LDL and high-sensitivity C-reactive protein were reached (388). Importantly, however, there was very little relation between the reductions in LDL and high-sensitivity C-reactive protein, with less than 2% of the variance in achieved high-sensitivity C-reactive protein explained by the variance in achieved LDL (388). These results suggest that the reduction in high-sensitivity C-reactive protein was likely cholesterol-independent.

As with outcome studies, the fact that LDL reduction by LDL apheresis or diet modification has been shown to improve endothelial function in humans suggests that observed improvements in vasomotor responses cannot be definitively attributed to the cholesterol-independent actions of the HMG-CoA reductase inhibitors (389,390). Additionally, most, but not all, studies investigating the various cholesterol-independent actions of the HMG-CoA reductase inhibitors in vitro and in animal models have employed doses well above those that are achieved with clinically relevant doses. Nonetheless, there is evidence to suggest that endothelial benefits
associated with the HMG-CoA reductase inhibitors extend beyond cholesterol reduction and possibly, contribute to their clinical benefit. Importantly, it appears that there is a differing time course between the vascular effects of the HMG-CoA reductase inhibitors vs. lipid lowering. Indeed, numerous studies have observed a rapid improvement in endothelial vasomotor responses as early as 24 hours after administration, prior to any change in plasma lipid levels (391-396). Evidence for the cholesterol-independent actions of the HMG-CoA reductase inhibitors on the endothelium in humans is also provided by studies comparing these agents to the cholesterol-lowering drug ezetimibe, which acts to reduce intestinal absorption of cholesterol. HMG-CoA reductase inhibitor administration significantly improved both conduit and resistance vessel vasomotor responses when compared to ezetimibe, despite similar reductions in plasma LDL values (369,397). Further, studies comparing low-dose HMG-CoA reductase inhibitor plus ezetimibe with high-dose HMG-CoA reductase inhibitor have demonstrated greater increases in FMD with high dose HMG-CoA reductase inhibitor therapy despite similar reductions in LDL (398,399), although this has not been consistently demonstrated (400).

1.3.3.4. **HMG-CoA reductase inhibitors and protection from IR injury**

Animal models of IR injury have consistently demonstrated that acute administration of HMG-CoA reductase inhibitors confers protection against IR injury, limiting infarct size and preserving functional recovery. Such protection against IR injury has been observed in studies involving pitavastatin and cerivastatin (401), simvastatin (384,402,403), pravastatin (404), rosuvastatin (405,406) and atorvastatin (407,408). Similar to the setting of IPC, the mechanisms of this phenomenon appear to be complex (Figure 1.3). Studies in animals suggest that eNOS and/or iNOS are upregulated after HMG-CoA reductase inhibitor treatment (384,402-408), and that the subsequent increase in NO bioavailability activates mKATP channels (295), which, as described above, play a central role in mediating cardioprotection. Additionally, activation of the reperfusion injury salvage kinase pathway has been demonstrated following HMG-CoA reductase inhibitor treatment (409). Although it remains unclear how HMG-CoA reductase inhibitors activate this pathway, it may be through the upregulation of adenosine production by the ecto-5’-nucleotidase enzyme (409). Other lines of evidence have demonstrated the importance of vasoactive prostaglandin production on the cardioprotective response of the HMG-CoA reductase inhibitors (410-414), a phenomenon that might also be dependent on both...
eNOS and iNOS (411,413). Consistent with this, HMG-CoA reductase inhibitors have been shown to upregulate many enzymes involved in the synthesis of prostaglandins, including cytosolic phospholipase A₂, COX-2, PGI₂ synthase, and prostaglandin E₂ synthase (410,411). As with mKₐ₅₆ activation, the upregulation of prostaglandin production by COX-2 has been shown to be both eNOS- and iNOS-dependent (411,413).

Animal models have similarly demonstrated the ability of HMG-CoA reductase inhibitors to protect the endothelium when administered prior to IR. Indeed, pretreatment with HMG-CoA reductase inhibitors preserves ACh-induced vasodilatory responses (415) and preserves coronary blood flow following reperfusion (403,416,417). Further, HMG-CoA reductase inhibitor pretreatment attenuates endothelial cell-leukocyte adhesion molecule expression (418,419), reducing leukocyte adhesion and vascular hyperpermeability (416,417), and preserves endothelial ultrastructural morphology following IR (416,417). As in the myocardium, bioavailable NO and mKₐ₅₆ channel activation appear essential for endothelial protection as co-administration with either a NOS inhibitor or mKₐ₅₆ channel blocker abolishes HMG CoA reductase inhibitor-mediated protection (403,416,417).

1.3.3.5. **Chronic HMG-CoA reductase inhibitor therapy and protection from IR injury**

To date, evidence as to whether this benefit afforded by acute HMG-CoA reductase inhibitor administration in the setting of IR is maintained with chronic therapy remains controversial. Animal models of chronic therapy with these agents have involved the use of simvastatin, pravastatin, atorvastatin and rosuvastatin and have produced mixed results with respect to their effect on IR (415,416,420-422); while there are some reports of sustained protection (415,416,421), other studies have reported a loss of protection with chronic therapy (415,416,420,422,423). Based on these reports, it appeared that the protective effect of chronic therapy might be dose-dependent as studies that observed of protection from IR employed doses well above the upper limit of those used clinically (416,422). The ability of chronic HMG-CoA reductase inhibitor therapy, at clinically relevant doses, to directly protect against IR injury remains unexplored in humans.

1.3.3.6. **HMG-CoA reductase inhibitor therapy and protection from IR injury in humans**

A number of studies have demonstrated that HMG-CoA reductase inhibitors may reduce IR injury and improve outcome after ischemic events in humans. However, the hypothesis that this
Following acute HMG-CoA reductase inhibitor administration, an upregulation of eNOS is observed through increased eNOS mRNA half-life, and an increase in enzymatic activity by PI3K/Akt-mediated phosphorylation. This increase in eNOS activation is required for the downstream upregulation and activation of iNOS, which is believed to occur via a NO-mediated activation of a signal transduction mechanism involving the enzyme PKC-ε and the transcription factor nuclear factor-κB (NF-κB). COX-2 has also been identified as an essential mediator of the HMG-CoA reductase inhibitor cardioprotective response. Both eNOS and iNOS have been shown to be essential for the upregulation of COX-2, likely by S-nitrosylation of the enzyme. Prostaglandin E₂ (PGE₂) and/or PGI₂ appear to be the most likely products of COX-2 leading to the cardioprotective response as both have been demonstrated to cause opening of the mKₐ₅ₖ channel, however the exact interrelationships between mKₐ₅ₖ and the iNOS, COX-2, PGE₂ and PG1₂ signaling cascade are currently not clear. Importantly, this cardioprotective response can be prevented with the prior administration of a COX-2 inhibitor such as celecoxib. Figure published in: (424)
effect may depend upon preconditioning-like properties of these drugs, and the underlying mechanisms, remain untested. Retrospective studies have consistently shown that HMG-CoA reductase inhibitor administration can reduce cardiovascular morbidity and/or mortality in patients when administered prior to episodes of cardiac ischemia (425-434). This hypothesis has been further investigated in prospective randomized trials in the setting of percutaneous coronary interventions or coronary bypass surgery. In the study by Briguori et al. and in the Atorvastatin for Reduction of Myocardial Damage during Angioplasty trial, pretreatment with atorvastatin was associated with a significant decrease in the incidence of periprocedural myocardial infarction in patients undergoing percutaneous coronary intervention procedures (435,436). In a follow up to the Atorvastatin for Reduction of Myocardial Damage during Angioplasty trial, atorvastatin pretreatment was associated with a similar decrease in periprocedural injury and major adverse cardiac events at 30 days when acutely administered to patients presenting with non-ST elevation myocardial infarction (437). More recently, the NAPLES II trial demonstrated a significantly lower incidence of cardiac creatine kinase and troponin elevation in patients treated with a single high loading dose of atorvastatin (80 mg) 24 hours prior to elective percutaneous coronary intervention (438). In the setting of cardiac surgery, Mannacio et al. showed that rosuvastatin pre-treatment (20 mg for 7 days) was associated with a significantly lower increase in troponin I, myoglobin and the myocardial isoenzyme of creatine kinase in patients undergoing coronary artery bypass (439). Importantly, the beneficial effect of HMG-CoA reductase inhibitors in these studies was independent, and additive, to that of glycoprotein IIb/IIIa inhibitors, β-blockers, and angiotensin-converting enzyme inhibitors. The mechanisms responsible for these positive outcomes remain unclear.

1.3.3.7. HMG-CoA reductase inhibitors and the prevention of nitrate tolerance
Evidence of a preventative effect of HMG-CoA reductase inhibitors in the setting of nitrate tolerance is provided by 3 separate reports demonstrating the ability of pravastatin, atorvastatin, and rosuvastatin to prevent GTN tolerance and GTN-induced endothelial dysfunction in normocholesterolemic rats (440-442). In all 3 reports, HMG-CoA reductase inhibitor pretreatment preserved the vasodilatory effect of GTN in thoracic aortic rings. This preservation of GTN activity was demonstrated to be a NO-dependent process and was also related to a reduction in superoxide formation from NADPH oxidase (440-442). Importantly, HMG-CoA reductase inhibitor-pretreatment also prevented the development of GTN-induced endothelial
dysfunction in these rats, assessed by measuring the vasodilatory response to ACh (441). The authors also that the prevention of tolerance was cholesterol independent as the addition of mevalonate abolished the protective effect of HMG-CoA reductase inhibition on GTN vasodilatory responsiveness (442). The ability of HMG-CoA reductase inhibitors to prevent the development of tolerance and endothelial dysfunction with chronic GTN therapy in humans has not been investigated.

1.4. Scope and hypotheses of thesis

As mentioned in the above sections, there is growing recognition that endothelial dysfunction contributes to cardiovascular disease development and progression. Such evidence emphasizes the need for sensitive methodologies to evaluate the impact of risk factors and disease on endothelial function in humans. Additionally, strategies to prevent the development of endothelial dysfunction in settings such as IR injury and nitrate tolerance, which may contribute to a worsening of endothelial function in patients already characterized by endothelial dysfunction, may be of clinical relevance. The first major objective of this thesis is to compare different methods of FMD measurement with regards to their repeatability, NO-dependency, and their sensitivity in distinguishing healthy vasodilatory responses from those in the presence of cardiovascular risk factors or disease. The goal of this study is to determine the ideal method(s) for characterizing the vasodilatory response to a hyperemic stimulus in order to aid in future method standardization. The second major objective is to investigate the ability of HMG-CoA reductase inhibitor (specifically rosuvastatin and atorvastatin) pretreatment and coadministration to positively modify conduit and resistance vessel-endothelial responses in settings not necessarily associated with hyperlipidemia, IR injury and nitrate tolerance, in humans in vivo and to determine potential mechanisms behind such modifications.

1.4.1. Preview and hypothesis of Chapter 2

The first study of this thesis was designed to compare methods of FMD measurement with respect to the repeatability of the measures, their NO-dependence, and their sensitivity in distinguishing between normal and compromised endothelial responses in healthy volunteers, in individuals with risk factors for cardiovascular disease, and in cardiovascular disease patients. Specifically, the repeatability of FMD_{max-cont}, FMD_{60}, and time to-FMD_{max} measurements performed in healthy volunteers was assessed using intraclass correlation coefficient (ICC) and
Bland and Altman analysis. The NO-dependency of the 3 measures was determined by performing consecutive FMD measurements, first with an intra-arterial infusion of normal saline, followed by the NOS inhibitor L-NMMA. Finally, to compare the sensitivity of each measure to distinguish health from disease, FMD measurements were performed in cohorts of young and middle-aged healthy volunteers, young smokers, and patients with HTN, CAD, and HF. We hypothesized that time to-FMD max would differ between healthy subjects and those with cardiovascular disease or cardiovascular risk factors. As such, we hypothesized that it may provide an additional characterization of the dilatory response following distal cuff occlusion along with peak diameter measurement. We also hypothesized that any differences in time to-FMD max may compromise the sensitivity of FMD 60 and further emphasize the need for continuous measurement of arterial diameter.

1.4.2. Preview and hypothesis of Chapter 3
In chapter 3 we examined the effect of acute HMG-CoA reductase inhibitor administration on the development of IR-induced endothelial dysfunction in the human forearm. There is a large amount of animal data demonstrating the ability of various HMG-CoA reductase inhibitors to exert a preconditioning-like effect, decreasing infarct size, improving functional recovery, and preserving vascular function following IR, independent of changes in lipid levels (384,402-408). Animal studies have also emphasized the importance of the COX-2 enzyme in mediating the protective effects of the HMG-CoA reductase inhibitors. Less is known about whether a similar preconditioning-like phenomenon exists in humans and whether it is similarly COX-2 dependent. We assessed conduit artery endothelial function by FMD before and after local IR in healthy volunteers who received a single dose of rosvuastatin or placebo 24 hours prior. In a separate protocol, we assessed the effect of single dose rosvuastatin vs. placebo on conduit artery, IR-induced endothelial dysfunction in volunteers pretreated with the selective COX-2 inhibitor celecoxib. We hypothesized that single-dose rosvuastatin would protect against the development of IR-induced endothelial dysfunction, and that this would occur without changes in plasma cholesterol. We also hypothesized that pretreatment with the COX-2 inhibitor celecoxib would prevent the endothelial-protective effect of rosvuastatin following IR.

1.4.3. Preview and hypothesis of Chapter 4
The study described in chapter 4 followed from the findings of chapter 3 and evaluated the effect of sustained HMG-CoA reductase inhibitor administration on the development of conduit artery,
IR-induced endothelial dysfunction. There is a substantial amount of evidence concerning the protective effects of the HMG-CoA reductase inhibitors against IR injury with acute administration. In contrast, less is known about whether the preconditioning-like effects of HMG-CoA reductase inhibitors are preserved during sustained, daily administration. We therefore aimed to assess whether daily rosuvastatin administration for 21 days would preserve the endothelial-protective effect associated with acute administration on conduit artery, IR-induced endothelial dysfunction measured by FMD. We also aimed to determine whether any observed protection with daily administration is similarly dependent upon the COX-2 enzyme by co-administering celecoxib with rosuvastatin. Because animal studies demonstrating a loss of protection from IR employed doses above those that are used clinically, we hypothesized that protection against IR-induced endothelial dysfunction in the radial artery would be preserved with daily rosuvastatin administration at a clinically relevant dose. We also hypothesized that this protection would be abolished with the co-administration of celecoxib.

1.4.4. Preview and hypothesis of Chapter 5
In the final experimental chapter of this thesis, we evaluated the effect of atorvastatin co-administration of the development of GTN-induced endothelial dysfunction and tolerance. Animal models of continuous GTN administration have previously demonstrated the ability of HMG-CoA reductase inhibitors to prevent the development of tolerance to exogenous GTN as well as to preserve endothelium-dependent vasodilatory responses to ACh by a mechanism that was NO-dependent and antioxidant in nature (440-442). The protocol designed in this chapter was designed to investigate whether atorvastatin co-administration exerts a similar effect in humans. Healthy volunteers were administered transdermal GTN and placebo, transdermal GTN and atorvastatin, or atorvastatin alone for 7 days. Measures of endothelial vasomotor function in resistance vessels were obtained by venous-occlusion strain gauge plethysmography in response to increasing doses of intra-arterial ACh, co-infused first with saline and repeated with a co-infusion of the antioxidant vitamin C. The effect of atorvastatin on GTN tolerance was assessed by measuring the beat-to-beat systolic blood pressure responses to a single sublingual dose of GTN. We hypothesized that atorvastatin co-administration would modify or prevent the development of endothelial dysfunction, and tolerance associated with chronic GTN therapy and that this prevention would be related to a reduction in oxidative stress.
Chapter 2 Observations of time-based measures of flow-mediated dilation of forearm conduit arteries: Implications for the accurate assessment of endothelial function

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A. Liuni and M. C. Luca contributed equally to this work

A. Liuni and M.C. Luca conceived and designed research protocol, performed experiments, analyzed data, interpreted results of experiments, prepared figures, drafted manuscript, edited and revised manuscript.

M. Lisi, S. Dragoni, and G. di Stolfo performed experiments.

T. Gori conceived and designed research protocol, performed experiments, edited and revised manuscripts, approved final version of manuscript.

J.A. Mariani and A. Uxa performed brachial artery cannulations for L-NMMA studies.

J.D. Parker conceived and designed research protocol, edited and revised manuscripts, approved final version of manuscript.
2.1. Abstract

Endothelium-dependent flow-mediated dilation is measured as the increase in diameter of a conduit artery in response to reactive hyperemia, assessed either at a fixed time point (usually 60-seconds post-cuff deflation, FMD\textsubscript{60}), or as the maximal dilation during a 5-minute continuous, ECG-gated, measurement (FMD\textsubscript{max-cont}). Preliminary evidence suggests that the time between reactive hyperemia and peak dilation (time to-FMD\textsubscript{max}) may provide an additional index of endothelial health. We measured FMD\textsubscript{max-cont}, FMD\textsubscript{60}, and time to-FMD\textsubscript{max} in 30 young healthy volunteers, 22 healthy middle-aged adults, 16 smokers, 23 hypertensive, 40 coronary artery disease, and 22 heart failure patients. As previously reported, FMD\textsubscript{max-cont} was similar in healthy cohorts and was significantly blunted in smokers and all patient groups, whereas FMD\textsubscript{60} was significantly blunted only in heart failure patients. There was a wide within-group variability between measures of time to-FMD\textsubscript{max} with no significant difference between normals and patient groups. Intra-arterial infusion of the NOS inhibitor levo-N-monomethylarginine in 8 healthy subjects resulted in blunting of FMD\textsubscript{max-cont} (\(P<0.001\)) and FMD\textsubscript{60} (\(P=0.02\)) but not time to-FMD\textsubscript{max}. Both FMD\textsubscript{max-cont} and FMD\textsubscript{60} demonstrated good repeatability in 30 young healthy volunteers studied on 2 separate occasions (\(P<0.01\) for both) while time to-FMD\textsubscript{max} varied widely between visits (\(P=NS\)). In conclusion, although time to-FMD\textsubscript{max} does not appear to be a useful adjunctive measure of endothelial health, the use of continuous diameter measurements provides important data in the study of endothelial function in healthy subjects and patients with cardiovascular disease.
2.2. Introduction

FMD is a noninvasive technique used to evaluate the function of the vascular endothelium in peripheral conduit arteries. The concept of FMD is based on the observation that the induction of ischemia in a peripheral limb (usually the hand/forearm), followed by rapid deflation of the pneumatic cuff used to induce this ischemia, causes a sudden increase in blood flow (reactive hyperemia) in the conduit artery that provides blood to this territory. The ensuing sudden change in local shear stress causes endothelium-dependent production of a number of vasoactive substances, including NO (80,221). Since the ability of the vessel to respond to changes in shear stress is dependent upon an intact and healthy endothelium, the magnitude of this flow-induced vasodilatory response has been proposed as a surrogate measure of overall endothelial function. Importantly, FMD of the radial artery has been demonstrated to be mediated primarily by NO, such that the response observed can also indicate the degree of NO bioavailability (58), although this concept has been recently challenged (235).

Traditionally, FMD is calculated as the dilation induced by reactive hyperemia, expressed in millimeters or percent change from resting diameter at either 60 seconds (FMD$_{60}$) or within a relatively narrow time range following cuff deflation. Measured this way, FMD correlates with coronary endothelial function (215) and is blunted in a number of cardiovascular conditions, including CAD, HTN and HF as well as in preclinical subjects with risk factors for atherosclerosis (51,58,215). However, the use of a fixed timeframe for FMD measurements has recently been questioned with a study demonstrating that the arterial diameters observed at 60 seconds underestimated the ‘true’ maximal FMD response (272). Further, in an attempt to better characterize the capacity of the vascular endothelium to react to changes in shear stress, other parameters of the FMD response have been proposed, including the measurement of the time to peak dilation (i.e. time difference between the induction of reactive hyperemia and the peak dilatory response, time to-FMD$_{max}$, see Figure 1). Interestingly, Black et al. recently showed that time to-FMD$_{max}$ is significantly shorter in young as compared to healthy middle-aged volunteers (272).

The present study investigated: 1) whether there are differences in repeatability and NO-dependency between different measures of FMD; 2) whether time to-FMD$_{max}$ is modified during infusion of a NO synthase inhibitor, and whether it is a repeatable measure; and 3) whether the
method used to measure FMD influences the capacity to distinguish between healthy subjects and patients with CAD, HTN, HF or smokers.

2.3. Materials and Methods

The ethics committees of the Mount Sinai Hospital (Toronto), the University Medical Center Mainz and the University of Siena approved measurement of FMD in healthy volunteers and patients with cardiovascular disease and written informed consent was obtained in all cases. All studies were performed between 11 AM and 2 PM. Participants were in a fasted state for at least 6h prior to the study.

2.3.1. Measurement of arterial diameter and time to $FMD_{max}$

The methods for the assessment of FMD in our laboratory, as well as the repeatability of measurements, have been previously described in detail (318,319,443-445). Briefly, end-diastolic, ECG-gated, longitudinal, B-mode images of the artery 10-15 cm below the antecubital fossa were digitally acquired and stored for off-line analysis. Arterial diameter was recorded continuously for 1 minute before cuff inflation (resting diameter), during the period of distal cuff inflation (4´30") and for another 4´30” after wrist cuff deflation. Semi-automatic custom-designed software that allowed for human correction was employed to calculate the arterial diameter from the trailing edge to the leading edge of the interface between the intima and blood. Three parameters were considered:

(1) $FMD_{max-cont}$, i.e. the maximum percent increase in arterial diameter in the 4´30” following cuff deflation as compared to resting diameter.

(2) $FMD_{60}$, i.e. the percent increase in arterial diameter at 60 seconds after wrist cuff deflation compared to resting diameter.

(3) Time to-$FMD_{max}$, i.e. the time interval from wrist-cuff deflation to maximal arterial diameter (see Figure 2.1 for all).

All data were analyzed in a randomized, blinded fashion after FMD data files were coded by laboratory staff not involved in data acquisition or analysis. Shear rate was calculated (in sec$^{-1}$) as blood velocity/radial artery diameter (446).
Figure 2.1. Schematic representation of different parameters of endothelial function. FMD$_{\text{max-cont}}$: flow-mediated dilation calculated as maximum arterial vasodilation after cuff deflation (diameter measured continuously for 4’30” using ECG triggering).
2.3.2. **Repeatability of the methods**

For repeatability studies (variability between occasions), 30 healthy young volunteers (18-34 years of age) underwent measurement of FMD$_{max-cont}$, FMD$_{60}$, and time to-FMD$_{max}$ on 2 separate occasions separated by $\geq$ 24 hours. In these subjects, the exact location of the probe was marked on the skin allowing the same imaging site to be used during each of the measurement periods.

2.3.3. **The role of NO in FMD and time to-FMD$_{max}$**

FMD$_{max-cont}$, FMD$_{60}$, and time to-FMD$_{max}$ were measured during the intra-arterial infusion of normal saline, followed by a 30-minute washout period, and subsequently during infusion of the NO synthase inhibitor L-NMMA (8µmol/min) in 8 healthy male volunteers (18-25 years of age) (75). Infusions were started 10 minutes before radial artery diameter recording. Due to the long half-life of L-NMMA, the order of the infusions was not randomized. However, preliminary studies from our laboratory (data not shown) and others showed that FMD measurements can be repeated at an interval of 30-60 minutes without significant variability (447-449).

2.3.4. **Differences between variables in health and disease**

For the comparison of FMD$_{max-cont}$, FMD$_{60}$, and time to-FMD$_{max}$ between healthy subjects and patients with risk factors and/or cardiovascular disease, 30 young healthy volunteers (18-35 years of age, 6 women), 22 healthy middle-aged adults (40-64 years of age, 9 women), 16 smokers (25-32 years of age, 9 women), 23 patients with HTN (35-65 years of age), 40 patients with known CAD (50-72 years of age, 7 women), and 22 patients with chronic HF (52-83 years of age, 5 women) were studied. Healthy volunteers were lifelong non-smokers with a systolic blood pressure $<130$ mmHg and a diastolic blood pressure $<80$ mmHg. Smokers had a history of 5-10 cigarettes/day for 3-10 years. Patients with HTN had undergone 24-hour blood pressure monitoring which documented average systolic values $>140$ and diastolic values $>90$ mmHg and were studied at the time of diagnosis (i.e. before initiation of therapy). None of the healthy volunteers, smokers or patients with HTN were on treatment with any drug, including supplemental vitamins, none had a history of diabetes, and none had clinical evidence of coronary artery or peripheral artery disease. CAD patients had at least one stenosis $>70\%$ in one major coronary artery as shown by angiography and had been clinically stable for at least 1 month. Patients with HF had a left ventricular ejection fraction $\leq 35\%$. They presented with New York Heart Association class II-III symptoms and were on a stable regimen including
diuretics, angiotensin converting enzyme inhibitors and beta-blockers for at least 1 month. All patients abstained from vasoactive medications on the day of the studies. All patients in the middle-aged adult, smoking, HTN, and HF groups, as well as 27 CAD patients and 14 healthy volunteers, were included in previous studies testing endpoints not related to the present study or to time-domain endpoints of FMD (443,450).

2.3.5. Statistical analysis
Data are presented as mean ± SD. For repeatability studies, a coefficient of variation (defined as the standard deviation of the differences between paired values divided by the mean and divided by √2) and an intra-class correlation coefficient (a measure of both correlation and agreement across two sets of data) were calculated to compare consecutive FMD$_{\text{max-cont}}$, FMD$_{60}$, and time to-FMD$_{\text{max}}$. Bland and Altman curves were also constructed and a bias value (mean of the absolute differences between consecutive measurements) for each data point was calculated (451). One-way ANOVA was employed for analysis of differences in each individual endpoint between patient and control groups. Linear regression analysis was performed to determine the relationship between age and parameters of FMD. A p value of <0.05 was set as the threshold for significance. Sample size estimations were performed for FMD$_{\text{max-cont}}$ and FMD$_{60}$ using an α=0.05 and 1-β=0.8. For paired sample size estimations, the correlation coefficient between paired measurements was obtained from the L-NMMA study described above. SAS 9.2. (Cary, NC) was used for all statistical analyses.

2.4. Results

2.4.1. Repeatability of FMD$_{\text{max-cont}}$, FMD$_{60}$, and time to-FMD$_{\text{max}}$
Baseline blood flow (experiment 1: 16.9±16.9; experiment 2: 17.2±21.7 ml/min), percent increase in blood flow after reperfusion (experiment 1: 1236±897; experiment 2: 1343±1042 %), baseline shear rate (experiment 1: 20.5±17.0; experiment 2: 18.3±16.3 sec$^{-1}$) and shear rate after reperfusion (experiment 1: 184.5±56.5; experiment 2: 182.5±54.1 sec$^{-1}$) were similar between repeated FMD experiments. Reproducing previous findings from our laboratory (443) and others, we found FMD$_{\text{max-cont}}$ to be very repeatable between two consecutive experiments in the 30 young, healthy volunteers who underwent repeated FMD procedures ≥ 24 hours apart. The intra-class correlation coefficient was found to be 0.7 for FMD$_{\text{max-cont}}$ (Figure 2.2a, P<0.001).
Figure 2.2. FMD$_{\text{max-cont}}$, FMD$_{60}$, and time to-FMD$_{\text{max}}$ measurements for consecutive FMD procedures in healthy volunteers. FMD$_{\text{max-cont}}$ (2a), FMD$_{60}$ (2c), and time to-FMD$_{\text{max}}$ (2e) were evaluated for FMD experiments performed \( \geq \) 24 hours apart in the same subject. The curves illustrate the correlation between the FMD$_{\text{max-cont}}$, FMD$_{60}$, and time to-FMD$_{\text{max}}$ measurements (ICC=0.7, \( P<0.001 \); ICC=0.6, \( P<0.001 \); ICC=0.2 \( P=\text{NS} \), respectively). Figures 2b, 2d and 2f illustrate the Bland-Altman curves of repeatability. The average values of the two corresponding FMD$_{\text{max-cont}}$, FMD$_{60}$ and time to-FMD$_{\text{max}}$ values are plotted against the difference between the same values. The limits of agreement for FMD$_{\text{max-cont}}$ are between -3.9% and 3.8%. The mean FMD$_{\text{max-cont}}$ value is 6.4±2.6%. The mean FMD$_{60}$ value is 3.6±3.0%. The limits of agreement for FMD$_{60}$ are between -4.6% and 5.0%. The limits of agreement are between -95 and 85 seconds for time to-FMD$_{\text{max}}$. The mean time to-FMD$_{\text{max}}$ value is 59.3 ± 35.0 seconds.
The coefficient of variation for $FMD_{\text{max-cont}}$ was 15%. We found $FMD_{60}$ to be slightly less repeatable than $FMD_{\text{max-cont}}$: the intra-class correlation coefficient was in this case 0.6 (Figure 2.2c, $P<0.001$) and the coefficient of variation was 29%. In contrast, we found a large degree of variability in time to-FMD$_{\text{max}}$ measurements. There was a non-significant correlation between repeated measurements of this variable (Figure 2.2c, intra-class correlation coefficient=0.2, $P=0.1$). The coefficient of variation for these data was 35%. The Bland-Altman plot for repeated $FMD_{\text{max-cont}}$ values is shown in Figure 2.2b. The bias calculated in absolute differences was 1.4% with 95% limits of agreement between -3.9 and 3.8%. The Bland-Altman plot for $FMD_{60}$ is shown in Figure 2.2d. The absolute bias was found to be 1.9%, which is slightly higher than that of $FMD_{\text{max-cont}}$, with 95% limits of agreements between -4.6 and 5.0%. The Bland-Altman plot for time to-FMD$_{\text{max}}$ is shown in Figure 2.2f. The absolute bias was 34 seconds. The 95% limits of agreement were between -95 and 85 seconds, indicating that the time to-FMD$_{\text{max}}$ for the second experiment performed on a given subject could be up to 85 seconds longer or 95 seconds shorter than the time to-FMD$_{\text{max}}$ value from the first experiment on the same subject.

2.4.2. The role of NO in $FMD_{\text{max-cont}}$, $FMD_{60}$, and time to-FMD$_{\text{max}}$

Infusion of L-NMMA caused a significant blunting of baseline radial artery blood flow without altering resting diameter, resulting in a significant percent increase in blood flow after deflation (Table 2.1). $FMD_{60}$ and $FMD_{\text{max-cont}}$ were both blunted during L-NMMA compared to placebo (respectively, from 4.6±3.0 to 0.9±2.6 and from 7.2±2.1 to 2.7±1.9, corresponding to a mean blunting of 80.1% in $FMD_{60}$ and of 64.7% in $FMD_{\text{max-cont}}$). The impact of L-NMMA on the two variables was not statistically different. In contrast, L-NMMA had no effect on the time to-FMD$_{\text{max}}$ (Table 2.1, Fig. 2.3).

2.4.3. $FMD_{\text{max-cont}}$, $FMD_{60}$, and time to-FMD$_{\text{max}}$ measurements in healthy volunteers and patients with cardiovascular disease

In the CAD group, 35 patients also had HTN, 38 had hypercholesterolemia, and 15 had diabetes mellitus. None of them had systolic or diastolic HF. All CAD patients were taking aspirin. As previously published by our group and others, baseline blood flow, blood flow after cuff inflation and reactive hyperemia were similar in all groups. $FMD_{\text{max-cont}}$ was similar between healthy young and healthy middle-aged volunteers and it was significantly blunted in all patient groups (Healthy, young: 7.3±3.0%; Healthy, middle aged: 6.1±4.3%; Smokers: 4.2±4.7%; HTN:
Table 2.1. Radial artery diameters and blood flow changes during saline and L-NMMA infusions

<table>
<thead>
<tr>
<th></th>
<th>Normal Saline</th>
<th>L-NMMA</th>
<th>P</th>
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<tbody>
<tr>
<td><strong>Radial Artery Diameter, mm</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting diameter</td>
<td>2.42±0.32</td>
<td>2.42±0.32</td>
<td>NS</td>
</tr>
<tr>
<td>Maximum change in diameter post-deflation</td>
<td>0.17±0.04</td>
<td>0.06±0.04</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Change in diameter at 60 seconds post-deflation</td>
<td>0.11±0.07</td>
<td>0.02±0.07</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Time to-FMD$_{max}$, seconds</td>
<td>61±22</td>
<td>91±67</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Radial Artery Blood Flow</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting blood flow, mL/min</td>
<td>18±11</td>
<td>9±5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Increase in blood flow after cuff deflation, %</td>
<td>843±307</td>
<td>1631±620</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

NS, not significant.
Figure 2.3. FMD_{max-cont}, FMD_{60}, and time to FMD_{max} measurements during the infusion of NS and L-NMMA. Values of FMD_{max-cont} were significantly lower following infusion of L-NMMA (P<0.001). FMD_{60} measurements were also significantly lower following L-NMMA infusion (P=0.02). Measurements of time to FMD_{max} were not significantly different after L-NMMA infusion.

NS, normal saline.
3.2±2.0%; CAD: 3.6±2.5%; HF: 2.7±3.6%; \( P<0.01 \) for all patient groups). FMD\(_{60}\) values were similar in the normal volunteer groups (healthy young and middle-aged) but were blunted in all patient groups (Healthy, young: 4.8±3.9%; Healthy, middle aged: 3.5±4.8%; Smokers: 1.9±5.0%; HTN: 1.3±2.5%; CAD: 2.0±2.5%; HF: 0.5±3.4%). The differences in FMD\(_{60}\) between the healthy middle-aged cohort and the various patient groups, however, reached statistical significance only in the case of the HF group (\( P<0.001 \) compared to healthy middle-aged volunteers). The time to FMD\(_{\text{max}}\) was not found to be significantly different between any of the groups (Healthy, young: 61.4±34.0 seconds; Healthy, middle aged: 75.9±33.0 seconds; Smokers: 59.0±29.6 seconds; HTN: 73.2±34.7 seconds; CAD: 64.4±45.0 seconds; HF: 86.0±42.8 seconds). In healthy volunteers, regression analysis showed a weak but statistically significant linear correlation between age and time to FMD\(_{\text{max}}\) (Figure 2.4). There was no relationship between age and the other parameters of FMD.

2.4.4. **Heterogeneity of time to FMD\(_{\text{max}}\) measurements**

In young healthy subjects and healthy middle-aged adults, time to FMD\(_{\text{max}}\) varied between 17-143 seconds and between 30-143 seconds respectively. The time to FMD\(_{\text{max}}\) in smokers ranged 22-134 seconds. In patients with HTN, CAD, and HF, the time to FMD\(_{\text{max}}\) values ranged from 26-150, 14-187, and from 13-172 seconds, respectively.

2.5. **Discussion**

FMD has been previously expressed as the percentage increase in conduit artery diameter at 60 seconds following induction of local reactive hyperemia as compared to resting conditions. The prognostic impact of such “fixed time-point” measurements of FMD has been clearly established (253,261,262,277). More recent investigations have called into question the use of measurements at a fixed time point, which may underestimate the true maximal dilatory response (272). A number of other parameters can be derived from the analysis of the response of a conduit artery to reactive hyperemia. Among these, recent studies have focused on the time course of the FMD response, and particularly on the measurement of the time from deflation of the pneumatic cuff to peak arterial diameter (time to FMD\(_{\text{max}}\)). However, it remains unclear whether this “time domain” parameter is, like other measures of FMD, determined by modifications in the endothelial release of NO, and whether it might complement the information from traditional “space domain” arterial diameter measurements of FMD. The present study
Figure 2.4. Regression analysis for time to-FMD$_\text{max}$ and age in healthy volunteers. Neither FMD$_{\text{max-cont}}$ values nor FMD$_{60}$ were significantly related to age. In contrast, time to-FMD$_\text{max}$ had a significantly positive correlation with age (p<0.05).
tested whether assessment of maximal FMD, calculated by continuous (ECG-gated) measurement of conduit arterial diameter (a parameter we have termed FMD\textsubscript{max-cont}), might result in a more robust measurement of this response.

2.5.1. Repeatability of FMD measures

Our data demonstrate that while FMD\textsubscript{60} was found to correlate well across consecutive measurements, it was slightly less repeatable than FMD\textsubscript{max-cont}. Using means, standard deviations and correlation coefficients acquired in the current study, we were able to construct sample size estimates for FMD\textsubscript{max-cont} and FMD\textsubscript{60} (Table 2.2). The number of subjects to be included per group for crossover and parallel studies are shown and further emphasize the lower sensitivity of FMD\textsubscript{60} compared to FMD\textsubscript{max-cont}. These calculations suggest that recording of arterial diameter for a broader time range after cuff deflation may further improve the predictive value of FMD.

In contrast to the high repeatability of FMD\textsubscript{max-cont} and FMD\textsubscript{60}, our data demonstrate a definite lack of correlation as well as an absence of repeatability between consecutive time to-FMD\textsubscript{max} values measured in healthy subjects (Figures 2.2c and 2.2d). As well, Bland and Altman plots show that the difference between corresponding measurements is in several cases larger than the mean of these measurements, indicating a lack of agreement between consecutive measurements. Taken together, these data question the reliability and clinical applicability of time to-FMD\textsubscript{max} and emphasize the advantage of prolonged arterial diameter measurement.

2.5.2. The mechanism of FMD responses

The mechanisms underlying FMD are fairly well known and have been previously described in detail (225). Using the model employed in the current study, studies have shown that the dilatory response that follows cuff deflation is NO-dependent with little effect of other known vasoactive mediators such as PGI\textsubscript{2} and EDHFs (58,233,443), recognizing that some controversy exists (235). Further, the kinetics of NO release are also interesting: while early vasodilation caused by sudden increases in shear stress is caused by Ca\textsuperscript{2+}-dependent activation of NO synthase, a sustained shear stress stimulus causes phosphorylation of the enzyme and possibly also recruits endothelium-independent mechanisms, thereby modifying the mechanisms of vasodilation (225). Thus, measurements of FMD at different time points may reflect different molecular mechanisms, and it remains unclear whether a threshold time exists where the dilation
Table 2.2. Sample size estimates for $\text{FMD}_{\text{max-cont}}$ and $\text{FMD}_{60}$

<table>
<thead>
<tr>
<th>Effect Size (% of baseline)</th>
<th>Crossover Design</th>
<th>Parallel Design</th>
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<tbody>
<tr>
<td>10</td>
<td>138</td>
<td>1636</td>
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<td>106</td>
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<td>50</td>
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<td>60</td>
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</table>
induced by hyperemia ceases to be endothelium- (or NO-) dependent. These considerations are further complicated by the presence of disease, which might modify the balance among these endothelial and non-endothelial mechanisms. In our model (4.5 minutes of occlusion distal to the artery studied), FMD\textsubscript{max-cont} occurred most frequently within the first two minutes of cuff deflation. NO synthesis inhibition with L-NMMA markedly reduced FMD\textsubscript{max-cont} and FMD\textsubscript{60} responses (demonstrating that both parameters are NO-dependent); however, it did not alter time to-FMD\textsubscript{max} (i.e. while L-NMMA blunted both FMD\textsubscript{max-cont} and FMD\textsubscript{60} in each patient, it absolutely did not modify time to-FMD\textsubscript{max}). This might suggest that while NO plays an essential role in mediating the overall dilatory response, it does not affect the time required to respond. Since patients with cardiovascular risk factors and disease are known to have impaired endothelial function, a phenomenon believed to be related to limited NO bioavailability, the lack of NO-dependency in the time to-FMD\textsubscript{max} parameter may explain its inability to discriminate health from disease. A small but statistically significant correlation was found between age and time to-FMD\textsubscript{max}, and future studies will need to test whether this relationship is maintained in larger populations. Black et al. demonstrated a significant difference between time to-FMD\textsubscript{max} values in cohorts of young and older healthy subjects, suggesting that this observation may have been due to the change in arterial compliance that accompanies advanced age (272). If confirmed, these data might support the concept that an increased vascular stiffness might “delay”, but not blunt, FMD. These hypotheses deserve further investigations.

2.5.3. Within-group heterogeneity of time to-FMD\textsubscript{max}

Our data show that time to-FMD\textsubscript{max} is highly heterogeneous in healthy subjects as well as smokers and patients with cardiovascular disease (HTN, CAD, HF). These data, which expand on the previous findings of Black et al. (272) in healthy subjects and those of Pálinkás et al. (273) in patients with CAD, indicate that the peak vasodilatory response occurs over a broad time period and suggest that the use of a limited time period for the measurement of arterial diameter (e.g. FMD\textsubscript{60}) systematically underestimates the response to the hyperemic stimulus, capturing the peak FMD response in very few individuals (Figure 2.3). Thus, our data suggest that continuous (ECG gated) analysis of arterial diameter after cuff deflation is able to detect the “true” peak FMD, improving the signal to noise ratio of the method.

2.5.4. Between-group comparisons of FMD\textsubscript{max-cont}, FMD\textsubscript{60}, and time to-FMD\textsubscript{max}

It has been well established that endothelium-mediated responses such as FMD are significantly
lower in patients with cardiovascular disease as well as in subjects with risk factors for atherosclerosis such as smoking and hypercholesterolemia (46,51,58,80). Our data indicate that while the peak vasomotor response to shear stress-mediated release of endothelial vasoactive mediators is impaired, the temporal kinetics of this response are highly variable and not different in the setting of health versus those who smoke or have overt cardiovascular disease. In an analysis of low-risk subjects, defined by a 10-year Framingham risk score of less than 10%, Chironi et al. found that time to-FMD_{max} was not associated with Framingham risk score or carotid artery intima-media thickness (276). Donald et al. similarly demonstrated no difference in time to-FMD_{max} between healthy control subjects and patients with type 2 diabetes mellitus or hypercholesterolemia (274). Our findings complement these observations, as they are the first to demonstrate that time to-FMD_{max} is not altered in the setting of smoking, HTN, CAD or HF and is not a robust measure of endothelial health and cardiovascular status. The large heterogeneity of time to-FMD_{max} also appears to play a role in the reduced sensitivity of FMD_{60} in detecting alterations in smokers, HTN, CAD, and HF compared to the measurement of FMD_{max-cont}. While FMD_{60} and FMD_{max-cont} both were lower in the disease groups, in the case of FMD_{60} (age adjusted) statistical significance was reached only in HF patients, a difference that is compatible with the high variability in the time kinetics of the vasodilatory response. A number of limitations need to be acknowledged, such as the relatively small sample size and the use of a specific FMD setup (ECG gating, radial versus brachial or coronary arteries, distal versus proximal cuff, etc). Also, since our goal was to compare different measures of FMD, we did not assess GTN-induced dilation, thus it is possible that impaired smooth muscle responsiveness may have contributed to our observed differences between healthy subjects and patients populations studied.

Our results demonstrate that time to-FMD_{max} is unable to differentiate between healthy controls and those with compromised cardiovascular health, and that, unlike other FMD measures, is not influenced by NO synthase blockade. This observation suggests that time to-FMD_{max} does not provide additional information that is complementary to “traditional” FMD in studying endothelial function in patients with cardiovascular risk factors or overt cardiovascular disease. Further, the heterogeneity of time to-FMD_{max} measures across healthy volunteers and patients with cardiovascular disease increases the variability and decreases the sensitivity of FMD_{60} in detecting differences in patient cohorts, as shown by our power calculations. Collectively, our
data emphasize the importance of prolonged measurement of arterial diameter throughout the entire FMD procedure.
Chapter 3 Rosuvastatin Prevents Conduit Artery Endothelial Dysfunction Induced by Ischemia and Reperfusion by a COX-2-Dependent Mechanism

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A version of this chapter is published in: J Am Coll Cardiol 2010;55:1002-6.

A. Liuni conceived and designed research protocol, performed experiments, analyzed data, interpreted results of experiments, prepared figures, drafted manuscript, edited and revised manuscript.

M.C. Luca performed experiments, edited and revised manuscripts.

T. Gori conceived and designed research protocol, edited and revised manuscripts, approved final version of manuscript.

J.D. Parker conceived and designed research protocol, edited and revised manuscripts, approved final version of manuscript.
3.1. Abstract

**Objectives:** To determine if single dose rosuvastatin (40mg) protects against ischemia and reperfusion (IR)-induced endothelial dysfunction in humans and whether this effect is cyclooxygenase-2 dependent.

**Background:** Animal studies have demonstrated that rosuvastatin can limit damage and improve recovery after IR, an effect that may be mediated by the lipid-independent activation of cyclooxygenase-2.

**Methods:** In a double-blind, parallel design, 20 volunteers were randomized to a single dose of oral rosuvastatin (40mg) or placebo. Twenty-four hours later, endothelium-dependent, flow-mediated dilation (FMD) of the radial artery was measured before and after IR (15 minutes of upper-arm ischemia followed by 15 minutes of reperfusion). In a separate protocol, 18 volunteers received the cyclooxygenase-2 inhibitor celecoxib (200mg BID p.o.) for 5 days. On day 4, subjects were randomized to single dose rosuvastatin (40mg) or placebo and 24-hours later underwent the same protocol as above.

**Results:** Pre-IR FMD was similar between groups. IR significantly blunted FMD in the placebo group (FMD pre-IR: 6.4±1.4%; post-IR: 1.1±3.8%, \( P=0.002 \)). Rosuvastatin prevented this impairment (FMD pre-IR: 7.5±3.1%; post-IR: 6.2±3.9%, \( P=NS \) versus rosuvastatin pre-IR; \( P=0.03 \) versus placebo). Pretreatment with celecoxib completely abolished rosuvastatin’s protective effect (FMD pre-IR: 8.0±2.2%; post-IR: 1.4±2.0%, \( P<0.001 \) compared with pre-IR, \( P=NS \) versus placebo, \( P=0.002 \) versus rosuvastatin alone).

**Conclusions:** Rosuvastatin pharmacologically prevents the development of IR-induced conduit artery endothelial dysfunction. This beneficial effect of rosuvastatin is mediated by a cyclooxygenase-2-dependent mechanism, evidence that may also provide potential mechanistic insight into the reported cardiotoxic effects of cyclooxygenase-2 inhibitors.
3.2. Introduction

The goal of therapy in patients with a myocardial infarction is timely and effective reperfusion to the infarcted area. Unfortunately, reperfusion itself can contribute to myocardial damage, a phenomenon called IR injury (84,85). Importantly, endothelial cells are particularly susceptible to, and actively participate in this IR injury(101,102). Damaged and dysfunctional endothelium reduces perfusion to areas of prior ischemia and can exacerbate tissue injury, contributing to subsequent organ damage (73). Thus, the endothelium is a major determinant of the capacity of a tissue to recover from IR, and interventions capable of protecting the endothelium from IR should be considered of direct clinical interest.

Exposure to brief periods of ischemia (IPC) can reduce myocardial and vascular sensitivity to IR-induced injury (73,282). Importantly, studies have demonstrated that certain pharmacological agents that target important effectors of the ischemic preconditioning pathway can mimic this phenotype (290,452): agents such as sildenafil (318), and GTN (319) have been shown to prevent IR-induced endothelial damage, an effect termed “pharmacologic preconditioning”. HMG-CoA reductase inhibitors have also been demonstrated to have such a preconditioning response in animal models (409,414,453,454). This protective phenotype has been shown to involve several mechanisms and mediators, including upregulation of the COX-2 enzyme (410,411,413). Whether a similar protective phenotype occurs in humans in vivo, and whether its manifestation is COX-2-dependent, remains uninvestigated.

3.3. Methods

The Mount Sinai Hospital Research Ethics Board approved this investigator initiated, non-industry funded study, and all subjects gave informed consent. Study procedures are described in detail in previous publications from our group (318,319) and in appendices 2 and 3.

3.3.1. Protocol 1: Effect of rosuvastatin on IR-induced conduit artery endothelial dysfunction

Twenty healthy nonsmoking volunteers (18 to 33 years old) were enrolled in a double-blind, randomized, placebo-controlled, parallel trial. On the first study visit standing blood pressure measurements were obtained followed by venous blood sampling for baseline lipid analysis. Subjects were then randomized to receive placebo or 40mg of rosuvastatin. Twenty-four hours after drug administration, standing blood pressure and plasma lipid measurements were repeated.
Subsequently, radial artery FMD was measured as previously described (318,319,455). After this measurement was completed, a pneumatic cuff placed above the antecubital fossa was inflated to 250 mmHg for 15 minutes to induce local ischemia. The cuff was then deflated, and 15 minutes of reperfusion were allowed before FMD was measured again. We elected not to test endothelium-independent vasodilators because previous studies have already demonstrated that this cycle of IR specifically impairs endothelium-dependent responses (73,318).

3.3.2. Protocol 2: Effect of celecoxib pretreatment
Eighteen healthy nonsmoking volunteers (18 to 33 years old) were enrolled in a double-blind, randomized, placebo-controlled parallel trial. After consent and baseline measurements as in protocol 1, subjects were administered 200mg BID of celecoxib, a selective COX-2 inhibitor, for 5 days. On day-4, subjects were randomized to receive a single dose of placebo or 40mg of rosuvastatin as in protocol 1. Twenty-four hours after randomization, subjects underwent FMD measurements before and after IR as described above.

3.3.3. Statistical Analysis
Data are presented as mean ± SD unless otherwise noted. Within-group comparisons were performed with a paired t-test. Between-group differences and the interaction of IR and randomization group were studied with a 2-way ANOVA. Post-hoc comparisons were performed using the Bonferroni correction. A value of $P<0.05$ was set as the threshold for significance. SAS 9.1.3. (SAS Institute Inc. Cary, NC) was employed for all statistical analyses.

3.4. Results

3.4.1. Effect of rosuvastatin and celecoxib administration on baseline parameters
There were no significant differences in resting blood pressure, resting radial artery diameter, baseline blood flow, reactive hyperemia, or pre-IR FMD among groups in either protocol (Table 3.1).

3.4.2. Protocol 1: Effect of placebo and rosuvastatin administration on IR-induced endothelial dysfunction
In both the placebo and the rosuvastatin group, resting radial artery diameter and radial artery blood flow returned to baseline values 15 minutes post-IR (Table 3.1). Similarly, peak reactive
Table 3.1. Arterial diameter and Blood Flow Data

<table>
<thead>
<tr>
<th>Radial Artery</th>
<th>Pre-IR</th>
<th>Post-IR</th>
</tr>
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<tbody>
<tr>
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<td></td>
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<td></td>
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</tr>
<tr>
<td><strong>PROTOCOL 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>2.38±0.32</td>
<td>0.15±0.03</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>2.29±0.25</td>
<td>0.17±0.02</td>
</tr>
<tr>
<td><strong>PROTOCOL 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Celecoxib+Placebo</td>
<td>2.43±0.20</td>
<td>0.20±0.06</td>
</tr>
<tr>
<td>Celecoxib+Rosuvastatin</td>
<td>2.48±0.26</td>
<td>0.20±0.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Blood Flow (mL/min)</th>
<th>Pre-IR</th>
<th>Post-IR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
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<td></td>
</tr>
<tr>
<td><strong>PROTOCOL 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>37.7±19.9</td>
<td>229.6±99.6</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>38.2±25.8</td>
<td>203.5±86.7</td>
</tr>
<tr>
<td><strong>PROTOCOL 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Celecoxib+Placebo</td>
<td>46.2±19.7</td>
<td>221.1±40.5</td>
</tr>
<tr>
<td>Celecoxib+Rosuvastatin</td>
<td>37.1±6.8</td>
<td>198.3±44.0</td>
</tr>
</tbody>
</table>

* P<0.01 versus corresponding value pre-IR.
† P<0.0001 versus corresponding value pre-IR.
‡ P=NS versus corresponding value pre-IR, P<0.05 versus FMD post-IR in the placebo group and versus both groups, protocol 2.
hyperemia was not significantly different post-IR (Table 3.1). IR significantly blunted FMD in the placebo group (Figure 3.1, pre-IR: 6.4±1.4%; post-IR: 1.1±3.8%, \(P=0.002\)). In contrast, rosvastatin administration prevented the impairment in FMD associated with IR (Figure 3.1, pre-IR: 7.5±3.1%; post-IR: 6.2±3.9%, \(P=\text{NS}\) versus rosvastatin pre-IR, \(P=0.002\) versus placebo, \(P=0.03\) for the interaction of IR and group).

3.4.3. **Protocol 2: Effect of celecoxib pretreatment on rosvastatin-mediated protection**

As in protocol 1, resting arterial diameter, radial artery blood flow and peak reactive hyperemia were not modified by IR in the celecoxib+placebo and the celecoxib+rosuvastatin groups (Table 3.1). IR significantly blunted FMD responses in the subjects who received celecoxib+placebo (Figure 3.2, pre-IR: 8.1±1.9%; post-IR: 2.2±2.8%, \(P<0.001\)). Similarly, in the subjects that received celecoxib+rosuvastatin, IR blunted FMD to values similar to those observed in the placebo group of protocol 1 (Figure 3.2, pre-IR: 8.0±2.2%; post-IR: 1.4±2.0%; \(P<0.001\) vs. pre-IR, \(P=\text{NS}\) versus celecoxib+placebo group, \(P=0.002\) for overall group effect, \(P=0.004\) for the interaction of IR and group, \(P=0.002\) versus rosvastatin group in protocol 1).

3.4.4. **Effect of rosvastatin and celecoxib administration on lipid parameters**

There were no significant differences in total cholesterol, high-density lipoprotein, LDL, or triglycerides between groups nor was there a significant difference in lipid parameters after the 24-hour period of treatment with rosvastatin, celecoxib, or placebo in either protocol (Table 3.2).

3.5. **Discussion**

The present study demonstrates, for the first time in humans, the ability of the HMG-CoA reductase inhibitor rosvastatin to create an endothelial pharmacological preconditioning effect in the setting of IR injury at the level of the conduit vasculature. This effect appears to be mediated by a COX-2 dependent mechanism and is independent of reductions in plasma lipids. Our results are consistent with evidence from animal models of ischemic injury, which have consistently demonstrated the ability of HMG-CoA reductase inhibitors to decrease infarct size, maintain vascular function, and improve functional recovery after IR injury (384,405-407,453,454).

We observed a significant blunting of FMD after IR in the placebo group, whereas the
FMD responses pre- and post-IR. In the placebo group, FMD was significantly blunted post-IR. This effect was prevented with rosuvastatin administration.

Data are mean ± SEM. *P=0.002 versus FMD before IR.
Figure 3.2. FMD Before and After IR in Celecoxib+Placebo and Celecoxib+Rosuvastatin Groups

FMD responses pre- and post-IR. In both groups, FMD was significantly attenuated post-IR demonstrating the inhibitory action of celecoxib on rosuvastatin-mediated protection.

Data are mean ± SEM. *P<0.001 versus corresponding FMD before IR. †P=0.002 versus rosuvastatin group in protocol 1, ANOVA results shown in text.
Table 3.2. Analysis of lipid parameters

<table>
<thead>
<tr>
<th></th>
<th>First Visit</th>
<th></th>
<th></th>
<th></th>
<th>Final Visit</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total Cholesterol</td>
<td>HDL</td>
<td>LDL</td>
<td>Triglycerides</td>
<td>Total Cholesterol</td>
<td>HDL</td>
<td>LDL</td>
</tr>
<tr>
<td><strong>PROTOCOL 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td>4.25±0.79</td>
<td>1.47±0.43</td>
<td>2.27±0.69</td>
<td>0.96±0.38</td>
<td>4.04±0.68</td>
<td>1.40±0.38</td>
<td>2.11±0.57</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td></td>
<td>4.19±0.52</td>
<td>1.67±0.59</td>
<td>2.00±0.49</td>
<td>1.18±0.68</td>
<td>4.16±0.49</td>
<td>1.64±0.57</td>
<td>1.9±0.57</td>
</tr>
<tr>
<td><strong>PROTOCOL 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Celecoxib+ Placebo</td>
<td></td>
<td>4.14±0.69</td>
<td>1.56±0.48</td>
<td>2.15±0.55</td>
<td>0.82±0.34</td>
<td>3.95±0.69</td>
<td>1.50±0.46</td>
<td>2.11±0.66</td>
</tr>
<tr>
<td>Celecoxib+</td>
<td></td>
<td>3.94±0.81</td>
<td>1.51±0.49</td>
<td>2.10±0.57</td>
<td>0.74±0.39</td>
<td>4.03±0.87</td>
<td>1.49±0.44</td>
<td>2.14±0.60</td>
</tr>
</tbody>
</table>

Data in mmol/L.
HDL, high-density lipoprotein.
administration of rosuvastatin prevented this effect. Studies in animals suggest that the mechanism leading to such preconditioning-mimetic properties of HMG-CoA reductase inhibitors is likely multifactorial with upregulation in the activity of ecto-5’ nucleotidase (an enzyme responsible for the production of adenosine) and of the endothelial and inducible isoforms of NOS (401,406,411). In addition, specific importance has been given to the induction of COX-2 activity, which is believed to depend upon a HMG-CoA reductase inhibitor-mediated increase in NO bioavailability from both endothelial and inducible NOS sources (411,413). Our study demonstrates that the endothelial protection afforded by rosuvastatin is abolished in the presence of COX-2 inhibition, suggesting that COX-2 is at least partially involved in the signaling cascade leading to rosuvastatin-mediated protection in humans. To our knowledge, the current study represents the first demonstration of the importance of COX-2 in a human, in vivo preconditioning model. This observation may have further clinical implications, as it may contribute to the clarification of the mechanisms behind the observed increases in cardiovascular morbidity and mortality in patients receiving COX-2 inhibitors (456-458).

3.5.1. Study limitations
The fact that the present data were acquired in healthy volunteers and in a circulation that is different from the coronary circulation needs to be acknowledged. Additionally, the assessment of IR–induced endothelial dysfunction was limited to the conduit circulation and not to the distal microcirculation, a vascular bed that is also of importance in clinical IR injury. As mentioned above, previous studies have shown the IR injury employed here specifically impairs endothelium-dependent responses while leaving endothelium-independent reactivity unaltered (73). Therefore, the fact that reactive hyperemia, a predominantly endothelium-independent process (459), is unimpaired in the present paper should not be unexpected.

3.6. Conclusions
We demonstrate that rosuvastatin administration exerts potent endothelial protection against IR injury in conduit vessels, via activation of COX-2. These results represent the first human evidence of a direct endothelial pharmacological preconditioning effect by rosuvastatin and may provide a mechanistic explanation to previous observations from clinical settings (409,414). Further, our data suggest a possible mechanistic explanation for the negative cardiovascular side effects of COX-2 inhibitors observed in clinical trials (456-458).
Chapter 4 Daily Therapy with Rosuvastatin does not Provide Protection from Ischemia and Reperfusion-Injury in the Human Forearm

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From the \textsuperscript{1}Division of Cardiology, Mount Sinai and University Health Network Hospitals, Toronto, Canada; \textsuperscript{2}Department of Pharmacology and Toxicology, University of Toronto, Canada; \textsuperscript{3}Department of Cardiology, University of Mainz, Germany.

A version of this chapter to be published in: Am J Physiol Heart Circ Physiol 2012

A. Liuni conceived and designed research protocol, performed experiments, analyzed data, interpreted results of experiments, prepared figures, drafted manuscript, edited and revised manuscript.

M.C. Luca performed experiments, edited and revised manuscripts.

T. Gori conceived and designed research protocol, edited and revised manuscripts, approved final version of manuscript.

J.D. Parker conceived and designed research protocol, edited and revised manuscripts, approved final version of manuscript.
4.1. Abstract

Studies have demonstrated that the acute administration of HMG-CoA reductase inhibitors has protective effects in the setting of IR. Previously, we demonstrated that acute rosuvastatin prevented IR-induced endothelial dysfunction in humans through a cyclooxygenase-2-dependent mechanism. Whether chronic HMG-CoA reductase inhibitor administration provides similar protection remains controversial and is unknown in humans. Eighteen male volunteers were randomized to receive a single dose of rosuvastatin (20 mg) or placebo. Twenty-four hours later, endothelium-dependent, radial artery FMD was measured before and after IR (15’ of upper-arm ischemia followed by 15’ of reperfusion). In a separate protocol, 30 healthy volunteers were randomized to receive oral rosuvastatin (20 mg/day) and placebo, rosuvastatin and celecoxib (100 mg BID) or placebo alone; all for 21 days. Twenty-four hours after the final administration, FMD was measured before and after IR. Pre-IR FMD was similar between groups in both protocols. In the acute administration protocol, rosuvastatin significantly prevented the blunting of FMD with IR (FMD pre-IR: 8.4±1.3%; post-IR: 6.2±1.3%, P=0.01 ANOVA, treatment-group interaction). In the daily administration protocol, IR significantly blunted FMD in the placebo group (FMD pre-IR: 7.5±0.9%; post-IR: 3.3±0.7%, P<0.001). Chronic treatment with rosuvastatin did not modify this impairment (FMD pre-IR: 6.9±0.4%; post-IR: 1.6±1.0%, P<0.001, P=NS ANOVA, treatment-group interaction). Similarly, FMD responses post-IR in volunteers receiving rosuvastatin and celecoxib did not significantly differ from placebo (FMD pre-IR: 8.3±0.9%; post-IR: 2.1±0.8%, P<0.001, P=NS ANOVA, treatment-group interaction).

In contrast to acute administration, chronic rosuvastatin does not prevent the development of IR-induced endothelial dysfunction in normal humans.
4.2. Introduction

The HMG-CoA reductase inhibitors are potent cholesterol lowering agents and are among the most widely used medications in the treatment of hypercholesterolemia and prevention of coronary artery disease. HMG-CoA reductase inhibitors have been clearly established as effective strategies in the primary and secondary prevention of cardiovascular events (344,345,348,349). Importantly, results of many studies have suggested that the vascular benefits associated with HMG-CoA reductase inhibitor administration may extend beyond cholesterol reduction, as they are hypothesized to have cholesterol-independent or “pleiotropic” effects (352,387). One such effect is the preconditioning-like phenotype conferred by the HMG-CoA reductase inhibitors in the setting of IR injury. Animal models have consistently demonstrated that the acute administration of HMG-CoA reductase inhibitors provides protection from IR injury in the cerebral, mesenteric and cardiac circulation (409,414,453,454). Similar to the setting of ischemic preconditioning, this protective phenotype has been shown to be highly NO dependent and associated with up-regulation of the COX-2 enzyme (409-411,413,414). Our laboratory has recently confirmed these observations in a human model of IR injury (460).

While there appears to be no doubt concerning the protection afforded by their acute administration, it remains unclear whether the pharmacologic preconditioning effect of HMG-CoA reductase inhibitors in the setting of IR injury is maintained during sustained, daily administration, with animal models producing mixed results to date (415,416,420-422). With this in mind, we sought to determine whether the protective effects of acute rosvastatin administration on endothelial function are maintained with daily administration and further to determine whether such protection is COX-2-dependent.

4.3. Methods

The Mount Sinai Research Ethics Board approved this study, and all subjects gave informed consent prior to beginning the study. Studies were conducted in a quiet, temperature and humidity-controlled environment. All subjects were required to fast and abstain from caffeine for 14 hours prior to the study. Exclusion criteria included any active disease, the use of medications (including supplemental vitamins), as well as risk factors for cardiovascular disease such as hypertension, smoking, hypercholesterolemia, and a family history of premature cardiovascular disease.
4.3.1. **Effect of acute and chronic rosuvastatin on IR-induced endothelial dysfunction**

4.3.1.1. **Acute administration protocol**

Eighteen healthy volunteers were recruited in a double-blind, randomized, placebo-controlled parallel trial. After study admission, standing blood pressure measurements were obtained followed by venous blood sampling for baseline lipid analysis. Subjects were then randomized to receive a single dose of placebo or 20 mg of rosuvastatin. Twenty-four hours after randomization, standing blood pressure and plasma lipid measurements were repeated. Subsequently, radial artery FMD was measured as described below. After this measurement was completed, a pneumatic cuff placed above the elbow was inflated to 250 mmHg for 15 minutes to induce local ischemia. The cuff was then deflated, 15 minutes of reperfusion were allowed, and FMD was measured again. Our laboratory has had significant experience in the use of this experimental protocol that allows studying, in humans in vivo, IR-induced endothelial dysfunction and the therapeutic impact of preconditioning (318,443,461). We elected not to test endothelium-independent vasodilators because previous studies have demonstrated that this cycle of IR specifically impairs endothelium-dependent responses while leaving non-endothelium-dependent smooth muscle responsiveness unaltered (73).

4.3.1.2. **Daily administration protocol**

In a separate protocol, 30 healthy nonsmoking volunteers (18 to 29 years old) were enrolled in a double-blind, randomized, placebo-controlled trial of parallel design. After study admission, blood pressure measurements and venous sampling for baseline lipid analysis were obtained as above. Subjects were randomized to receive rosuvastatin (20mg/day) and placebo, rosuvastatin (20mg/day) and celecoxib (100mg/BID), or matching placebo. Subjects were then given a 20-day supply of the study medications and were discharged from the laboratory. Twenty-one days later, and 24 hours after the final doses of study medication, standing blood pressure and plasma lipid measurements were repeated. Subjects then underwent the same protocol of FMD measurements before and after IR, as described above.

4.3.2. **Measurement of arterial diameter and FMD**

The methods for assessment of FMD and blood flow in our laboratory, as well as the repeatability of FMD measurements, have been previously described in detail (318,319,443,461). Briefly, end-diastolic, ECG-gated, longitudinal, B-mode images of the artery 10-15 cm below the
antecubital fossa were digitally acquired and stored for off-line analysis. Arterial diameter was recorded continuously for 1 minute before cuff inflation (resting diameter), during the period of distal cuff inflation (4’30”) and for another 4 minutes and 30 seconds after wrist cuff deflation. Analysis was performed in a blinded fashion using automatic custom-designed vascular edge detection software (455). FMD was calculated as the maximum percent increase in arterial diameter following cuff deflation as compared to resting diameter.

4.3.3. Statistical Analysis

Data are presented as mean ± SEM. For our model of ischemic injury, sample size estimates were performed based on data from our recently published paper (460) using a 2-sided \( \alpha = 0.05 \) and \( 1 - \beta = 0.8 \). IR decreased FMD responses from 8.1±0.6% to 2.2±0.9%. Prevention of 50% of this impairment via rosuvastatin-induced preconditioning requires a sample size of 10 subjects per group. This sample size yields a \( 1 - \beta = 0.8 \), with a 2-sided \( \alpha = 0.05 \). Within-group comparisons were performed with a paired t-test. Between-group differences and the interaction of IR and randomization group were analyzed with a 2-way ANOVA. A value of \( P < 0.05 \) was set as the threshold for significance. SAS 9.2. (SAS Institute Inc. Cary, NC) was employed for all statistical analyses.

4.4. Results

4.4.1. Effect of rosuvastatin on baseline parameters

There were no significant differences in resting blood pressure (data not shown), resting radial artery diameter, baseline blood flow, reactive hyperemia, or FMD before IR between groups in either protocol (Table 4.1).

4.4.2. Acute administration protocol

4.4.2.1. Effect of acute rosuvastatin on IR-induced endothelial dysfunction

Resting radial artery diameter and radial artery blood flow returned to baseline values 15 minutes after local IR (Table 4.1, \( P = \text{NS} \), before versus after IR). Similarly, peak reactive hyperemia was not significantly different after IR. IR significantly blunted FMD in the placebo group (Figure
Table 4.1: Arterial diameter and blood flow data for acute administration protocol

<table>
<thead>
<tr>
<th>Radial Artery Diameter (mm)</th>
<th>Before IR</th>
<th>After IR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline Diameter</td>
<td>Change After Wrist Cuff Deflation</td>
</tr>
<tr>
<td>Placebo</td>
<td>2.28±0.07</td>
<td>0.20±0.02</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>2.37±0.11</td>
<td>0.20±0.03</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Blood Flow (mL/min)</th>
<th>Baseline</th>
<th>After Wrist Cuff Deflation</th>
<th>Reactive Hyperemia (%)</th>
<th>Baseline</th>
<th>After Wrist Cuff Deflation</th>
<th>Reactive Hyperemia (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>9.6±1.2</td>
<td>116.9±18.1</td>
<td>1164±178</td>
<td>9.4±1.1</td>
<td>124.4±18.6</td>
<td>1198±120</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>12.9±2.7</td>
<td>161.9±17.4</td>
<td>1424±251</td>
<td>11.0±1.7</td>
<td>181.3±19.2</td>
<td>1524±193</td>
</tr>
</tbody>
</table>

* P<0.001 vs. corresponding value before IR, † P<0.05 vs. corresponding value before IR.
4.1, pre-IR: 8.7±0.7%; post-IR: 2.3±1.3%, 95% confidence interval for change in FMD after IR: -8.372% to -4.433% \( P=0.002 \), confirming previous data of endothelial dysfunction induced by IR in this experimental protocol (318,319,443,444). Reproducing previous findings from our laboratory, rosuvastatin administration prevented the impairment in FMD associated with IR (Figure 4.1, pre-IR: 8.4±0.7%; post-IR: 6.2±3.9%, 95% confidence interval for change in FMD after IR: -4.171% to -0.2323%, \( P<0.05 \) versus rosuvastatin pre-IR, \( P=0.01 \) for the interaction of IR and group).

### 4.4.3. Sustained administration protocol

#### 4.4.3.1. Effect of daily rosuvastatin on IR-induced endothelial dysfunction

As with the acute administration protocol, resting radial artery diameter and radial artery blood flow and peak reactive hyperemia were not different from baseline 15 minutes after local IR (Table 4.2, \( P=NS \), before versus after IR). Similarly, IR significantly blunted FMD in the placebo group (Figure 4.2, before IR: 7.5±0.9%; after IR: 3.3±0.7%, 95% confidence interval for change in FMD after IR: -6.515% to -2.029%, \( P<0.001 \)). Importantly, chronic rosuvastatin administration did not prevent the impairment in FMD associated with IR (Figure 4.2, before IR: 6.9±0.4%; after IR: 1.6±1.0%, \( P<0.001 \) compared with rosuvastatin before IR, 95% confidence interval for change in FMD after IR: -7.564% to -3.078%, \( P=NS \) for ANOVA effect of group and for the interaction of IR and group). In subjects receiving rosuvastatin + celecoxib, IR blunted FMD to values similar to those observed in the placebo and rosuvastatin + placebo groups (Figure 4.2, before IR: 8.3±0.9%; after IR: 2.1±0.8%; \( P<0.001 \) compared with FMD before IR, 95% confidence interval for change in FMD after IR: -8.363% to -3.877%, \( P=NS \) for ANOVA effect of group and for the interaction of IR and group).

#### 4.4.4. Effect of rosuvastatin and celecoxib on lipid parameters

Results are summarized in table 4.3. No significant differences were noted in any lipid parameters after the 24-hour period of treatment in the acute administration protocol. In the daily administration protocol, significant decreases were observed in total cholesterol, LDL cholesterol, and triglyceride levels in rosuvastatin + placebo and rosuvastatin + celecoxib groups. There were no significant differences in lipid profiles between visits in the placebo group.
Figure 4.1. FMD responses before and after IR in acute administration protocol

FMD was significantly blunted post-IR in the placebo group. Single-dose rosuvastatin administration prevented this effect.

*P<0.001 versus FMD before IR, †P<0.05 versus FMD before IR.
Table 4.2: Arterial diameter and blood flow data for sustained administration protocol

<table>
<thead>
<tr>
<th>Radial Artery</th>
<th>Before IR</th>
<th>After IR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Change After Wrist Cuff Deflation</td>
</tr>
<tr>
<td></td>
<td>Diameter</td>
<td>Wrist Cuff Deflation</td>
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<tr>
<td>Placebo</td>
<td>2.26±0.14</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td>Rosuvastatin+Placebo</td>
<td>2.41±0.10</td>
<td>0.17±0.01</td>
</tr>
<tr>
<td>Rosuvastatin+Celecoxib</td>
<td>2.48±0.09</td>
<td>0.20±0.02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Blood Flow (mL/min)</th>
<th>Baseline</th>
<th>After Wrist Cuff Deflation</th>
<th>Reactive Hyperemia (%)</th>
<th>Baseline</th>
<th>After Wrist Cuff Deflation</th>
<th>Reactive Hyperemia (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>11.1±2.3</td>
<td>116.6±11.9</td>
<td>1169±157</td>
<td>10.7±2.2</td>
<td>117.9±12.9</td>
<td>1280±222</td>
</tr>
<tr>
<td>Rosuvastatin+Placebo</td>
<td>13.5±2.9</td>
<td>103.3±8.7</td>
<td>1057±229</td>
<td>11.1±1.9</td>
<td>103.2±8.1</td>
<td>1073±201</td>
</tr>
<tr>
<td>Rosuvastatin+Celecoxib</td>
<td>10.0±1.8</td>
<td>106.5±9.1</td>
<td>1036±95</td>
<td>10.7±2.2</td>
<td>102.5±7.0</td>
<td>1108±137</td>
</tr>
</tbody>
</table>

* P<0.001 vs. corresponding value before IR.
Figure 4.2. FMD responses before and after IR in sustained administration protocol

In both rosvastatin groups, FMD was significantly attenuated post-IR similar to placebo, demonstrating the lack of protection against IR-induced endothelial damage with chronic rosvastatin administration.

\*P<0.001 versus corresponding FMD before IR.
Table 4.3: Analysis of lipid parameters for acute and daily administration protocols

<table>
<thead>
<tr>
<th></th>
<th>First Visit</th>
<th></th>
<th>Second Visit</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total Cholesterol</td>
<td>HDL</td>
<td>LDL</td>
</tr>
<tr>
<td>Acute Administration protocol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>4.2±0.1</td>
<td>1.4±0.1</td>
<td>2.2±0.1</td>
<td>1.19±0.13</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>3.6±0.3</td>
<td>1.5±0.2</td>
<td>1.9±0.2</td>
<td>0.69±0.08</td>
</tr>
<tr>
<td>Daily Administration protocol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>4.0±0.3</td>
<td>1.7±0.2</td>
<td>2.1±0.3</td>
<td>0.97±0.14</td>
</tr>
<tr>
<td>Rosuvastatin+Placebo</td>
<td>4.2±0.2</td>
<td>1.6±0.2</td>
<td>2.2±0.2</td>
<td>1.03±0.68</td>
</tr>
<tr>
<td>Rosuvastatin+Celecoxib</td>
<td>4.0±0.2</td>
<td>1.4±0.1</td>
<td>2.2±0.2</td>
<td>0.85±0.13</td>
</tr>
</tbody>
</table>

Values in mmol/L.

* $P<0.01$ vs. corresponding value on visit 1.
† $P<0.05$ vs. corresponding value on visit 1.

HDL, high-density lipoprotein.
4.5. Discussion

HMG-CoA reductase inhibitors are among the most widely agents in the prevention and management of patients with CAD. Their long-term treatment benefits are unquestioned, although the spectrum of the mechanism(s) of their beneficial effects remains controversial. Although originally discovered and developed based on their lipid-lowering characteristics it is now documented that these agents possess cholesterol-independent effects that may play an important role in their beneficial effects in patients with risk factors or overt cardiovascular disease. These effects include increased activity of NOS through PI3K/Akt-mediated phosphorylation; an increase in the production of the essential NOS cofactor tetrahydrobiopterin as well as improved NOS mRNA stability, thus prolonging mRNA half-life for this important enzyme (376,462). Overall there is strong evidence that therapy with HMG-CoA reductase inhibitors is associated with an increase in NO bioavailability that is independent of reductions in plasma cholesterol (463). Such effects may play an important role in the improvement of outcomes in primary and secondary prevention studies with the HMG-CoA reductase inhibitors (344,345,348,349,387).

There is also considerable evidence that HMG-CoA reductase inhibitors are cardioprotective in the setting of ischemic injury. Animal models of low-flow ischemic injury in the brain, cardiac and mesenteric circulation have consistently demonstrated the ability of HMG-CoA reductase inhibitors, when administered acutely, to decrease infarct size, maintain vascular function, and improve functional recovery after IR injury in a pharmacologically-induced response similar to that of IPC (409,414,453,454). This response has been shown to be largely dependent upon NO and activation of downstream mediators, such as COX-2 (409,414). We recently confirmed the presence of a similar protective phenomenon following acute rosuvastatin administration in humans (460). Whether this benefit is maintained during chronic HMG-CoA reductase inhibitor administration, however, remains controversial. Animal models of chronic therapy with these agents have produced mixed results with respect to their effect on IR (415,416,420-422); while there are some reports of sustained protection (416,421), other studies have reported a loss of protection during sustained therapy (415,420,422).

In the present study, we observed that sustained therapy with rosuvastatin did not provide protection from the adverse effects of IR on endothelial function. These data are in contrast to
our current and previous data showing that acute rosuvastatin administration prevents the impairment in endothelium-dependent vasodilation associated with IR by a mechanism involving the COX-2 (424,460). Although prospective and retrospective studies in patients undergoing percutaneous coronary interventions, coronary artery bypass grafting, or experiencing acute coronary syndromes have consistently shown that acute HMG-CoA reductase inhibitor treatment can reduce evidence of myocardial injury as well as cardiovascular morbidity and/or mortality (409,414), the fact remains that most, if not all, patients continue with chronic statin therapy. The results of the current study suggest that the direct pharmacologic preconditioning, cardioprotective benefits of the HMG-CoA reductase inhibitors are lost with chronic therapy (464). Although there is no doubt that HMG-CoA reductase inhibitors are potent in both the treatment and prevention of CAD, the findings reported here suggest that a preconditioning effect does not play a role in their beneficial effects on long-term outcome.

The mechanisms behind the loss of direct cardioprotection with sustained HMG-CoA reductase inhibitor therapy are not well understood. It is possible that sustained exposure to an intervention (either pharmacologic or ischemic) with preconditioning effects could trigger counter-regulatory responses that lead to loss of protection from IR. Although there are many possible mechanisms through which this counter-regulation could occur, some important examples have already been defined. As mentioned above, the PI3K/Akt signaling cascade is a biochemical pathway that is of essential importance in the development of the preconditioning protective phenotype induced by acute treatment with HMG-CoA reductase inhibitors. Previous studies in animal models of IR injury have demonstrated that an increase in the levels of the PI3K inhibitor PTEN is associated with chronic treatment with HMG-CoA reductase inhibitors, leading to a downregulation of the PI3K/Akt pathway (422). The response of PTEN to preconditioning interventions is time dependent and provides a conceptually important explanation concerning differences between responses to acute and sustained preconditioning interventions. During acute exposure to HMG-CoA reductase inhibitors, PTEN is down regulated with resulting disinhibition of cell survival pathways, while during chronic exposure to the same stimulus, it is up-regulated, leading to loss of preconditioning effects (422). Despite the loss of this cardioprotective effect, the response is biologically appropriate since continued loss of PTEN’s tonic inhibitory effects can lead to inappropriate cellular proliferation (465).
Interestingly, we have recently reported a similar phenomenon (loss of pharmacologic preconditioning protection during prolonged administration) with daily short-term (2-hr) GTN administration in humans (339). This loss of cardioprotection contrasts with the effective preconditioning effects of a single short exposure to GTN (319,339,445). These lines of evidence serve to emphasize that the response to acute versus repeat pharmacologic stimuli can be very different and that an understanding of their underlying mechanism may be helpful in development of effective sustained preconditioning treatment strategies.

Of relevance, despite intense interest concerning the efficacy and mechanism of preconditioning interventions few investigations have explored whether preconditioning protection is sustained with repeated exposure. Two reports in a porcine myocardial infarction model have demonstrated that repeated IPC stimuli are associated with sustained protection from ischemia over 96 hours (466,467). Interestingly, these studies provided evidence to suggest that mechanism(s) responsible for the preserved protection with sustained IPC are different from those involved with acute IPC (466,467). Our current observations, although negative, emphasize the need to develop a better understanding of the mechanistic differences between acute and sustained preconditioning strategies in order to develop effective approaches that maintain a sustained preconditioned phenotype.

The fact that the present data were acquired in healthy volunteers and in the conduit circulation of the forearm, a vasculature different from the coronary circulation, is acknowledged as a limitation. However, the model employed in the present study has been previously shown to provide reliable and relevant information of the effect of IR injury on the vasculature. Study of the endothelium has particular relevance, since this tissue is the most sensitive to IR, and damage of the (macro- and microvascular) endothelium can prevent effective reperfusion in spite of timely intervention. For these reasons, we believe that the study of potential therapies and treatment regimens aimed at protecting the endothelium in the setting of IR are clinically relevant. Further studies should be conducted in individuals with dyslipidemia and patients with cardiovascular disease to determine whether a similar phenomenon exists in the presence of risk factors and/or disease. The small sample size in the present study needs to be acknowledged as a limitation. However, the possibility of a type II statistical error appears unlikely since absolutely no trend towards a protective effect of repeated rosuvastatin administration was shown, and the blunting in FMD in the rosuvastatin groups was actually slightly more pronounced than in the
placebo group. Finally, we cannot discount the possibility that a lack of compliance may have played a role in our observed responses. Although lipid parameters were significantly lowered in subjects receiving rosuvastatin, we acknowledge this as a limitation.

In conclusion, although it is clear that HMG-CoA reductase inhibitors are effective in the settings of both primary and secondary prevention the current study does not suggest that these favourable outcomes are, in part, due to a sustained preconditioning effect. The present data are in contrast to our previous report demonstrating the potent protective effects of acute rosuvastatin administration (460). The findings emphasize that an effective pharmacologic preconditioning stimulus induced by acute administration of a drug cannot be assumed to be associated with protection during repeated exposure.
Chapter 5 Co-administration of Atorvastatin prevents nitroglycerin-induced endothelial dysfunction and nitrate tolerance in healthy humans

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A version of this chapter is published in: J Am Coll Cardiol 2011;57:93-8.

A. Liuni conceived and designed research protocol, performed experiments, analyzed data, interpreted results of experiments, prepared figures, drafted manuscript, edited and revised manuscript.

M.C. Luca performed experiments, edited and revised manuscripts.

G. di Stolfo performed experiments.

A. Uxa, and J.A. Mariani performed brachial artery cannulations.

T. Gori conceived and designed research protocol, edited and revised manuscripts, approved final version of manuscript.

J.D. Parker conceived and designed research protocol, edited and revised manuscripts, approved final version of manuscript.
5.1. Abstract

Objectives: We aimed to assess whether concurrent administration of atorvastatin would modify the development of tolerance and endothelial dysfunction associated with sustained nitroglycerin (GTN) therapy in humans.

Background: Animal studies have demonstrated that administration of 3-hydroxy-3 methylglutaryl coenzyme A reductase inhibitors can protect against GTN-induced endothelial dysfunction and tolerance, likely through an antioxidant mechanism.

Methods: Thirty-six healthy male volunteers were randomized to receive continuous transdermal GTN (0.6 mg/h) and placebo, atorvastatin (80 mg/day) alone, or continuous transdermal GTN (0.6 mg/h) with concurrent atorvastatin (80 mg/day), all for 7 days. On the second visit, forearm blood flow was measured with venous-occlusion strain gauge plethysmography in response to incremental infusions of ACh (7.5, 15, and 30 µg/min). ACh infusions were co-infused first with saline, and repeated during the co-infusion of vitamin C (24 mg/min). Blood pressure responses to sublingual GTN (400 µg) were assessed on both visits.

Results: ACh responses in the GTN plus placebo group were significantly attenuated vs. those in the GTN plus atorvastatin and atorvastatin groups \( (P<0.01) \). Co-infusion of vitamin C completely restored ACh responses in the GTN plus placebo group \( (P<0.01 \text{ vs. saline co-infusion}) \) but caused no change in either the atorvastatin or the GTN plus atorvastatin groups. Blood pressure responses to sublingual GTN did not significantly change between visits in subjects receiving GTN plus atorvastatin and atorvastatin alone but were significantly blunted in the GTN plus placebo group \( (P<0.05) \).

Conclusions: The present findings demonstrate, for the first time in humans, that atorvastatin prevents both GTN-induced endothelial dysfunction and nitrate tolerance, likely by counteracting the GTN-induced increase in oxidative stress.
5.2. Introduction

GTN and other organic nitrates are widely used in the management of cardiovascular disease. The vascular effects of GTN are mediated by the release of NO or a NO-related species by denitrification of the nitrate ester (108). However, the clinical utility of chronic GTN therapy is limited by the rapid loss of the hemodynamic and anti-ischemic effects, a phenomenon termed tolerance (468). Both experimental and clinical observations indicate that an important cause of nitrate tolerance is an increase in the vascular bioavailability of ROS (146). Multiple sources of ROS have been described in response to nitrate therapy including NADPH oxidases, mitochondria, and uncoupled NOS (108). The increased bioavailability of ROS also results in the development of endothelial dysfunction, a consequence of sustained nitrate therapy that is now well documented (74,75,150). These observations have led to a number of new concepts concerning the etiology of tolerance, the role of nitrate biotransformation as a trigger of increased free radical production as well as the exploration of multiple therapeutic strategies to prevent the nitrate-induced increase in ROS (343,469). Recent observations in animal models have demonstrated that concurrent therapy with HMG-CoA reductase inhibitors can modify the development of tolerance and endothelial dysfunction associated with chronic GTN therapy by directly counteracting the GTN-induced increase in ROS (440-442). Whether HMG-CoA reductase inhibitor administration can preserve GTN responsiveness and prevent the development of GTN-induced endothelial dysfunction in humans, and whether a similar antioxidant mechanism is involved, has yet to be established.

5.3. Methods

The Mount Sinai Research Ethics Board approved this investigator initiated, non-industry funded study, and all subjects gave written informed consent prior to beginning the study. Studies were conducted in a quiet, temperature- and humidity-controlled environment.

5.3.1. Study Population

Thirty-six normal healthy, male, nonsmoking volunteers (18-27 years old) were enrolled in a randomized, parallel trial. All subjects were required to fast and abstain from caffeine for 14 hours before the study. Exclusion criteria included any active disease, the use of medications (including supplemental vitamins), as well as risk factors for cardiovascular disease such as
hypertension, smoking, a family history of premature cardiovascular disease, and hypercholesterolemia as defined by Canadian guidelines (470).

5.3.2. Study visit 1

After admission into the study, standing blood pressure and heart rate were measured using an automatic, calibrated sphygmomanometer (GE Healthcare, Mississauga, Ontario, Canada). Baseline FBF was then measured by forearm venous-occlusion strain gauge plethysmography, as described previously (75,150). Once these measurements were complete, subjects were administered 400 µg GTN as a sublingual spray and beat-to-beat blood pressure was monitored from the middle finger of the dominant hand using a finometer system (Finapres Medical Systems, Amsterdam, the Netherlands) for the next 4 minutes. Thirty minutes later, subjects were randomized to receive transdermal GTN 0.6 mg/h and atorvastatin (80mg/day), transdermal GTN 0.6 mg/h and matching placebo, or atorvastatin alone. Repeat standing blood pressure and heart rate measurements were taken 3 hours later. At the conclusion of visit 1, subjects were given a 6-day supply of either atorvastatin or placebo and were instructed to take one pill daily at 9 AM until the end of the study. Subjects assigned to GTN treatment were given a supply of transdermal GTN 0.6 mg/h for the following 6 days. Subjects were instructed to wear the patch continuously and to change it every morning at 9 AM. All oral medications (placebo and atorvastatin) were administered in a double-blind fashion while GTN patches were administered in an investigator-blinded fashion (in this case study personnel not involved in data acquisition or analysis were responsible for randomization procedures and answering any questions subjects had during the study).

5.3.3. Study visit 2

After 7 days of study medication, subjects returned to the laboratory for the assessment of 1) resistance artery endothelial function and tolerance, and 2) the role of oxidative stress in GTN- and atorvastatin-induced changes in endothelial responses. Standing blood pressure and heart rate measurements were repeated as described in visit 1. Once complete, the brachial artery of the non-dominant arm was cannulated as previously described (75). FBF was then measured during intra-arterial infusions of normal saline and in response to increasing concentrations of the endothelium-dependent vasodilator ACh as described previously (75,150). ACh was first co-infused with normal saline; ACh responses were subsequently repeated with a vitamin C co-infusion (24 mg/min) (150). When FBF measurements were complete, and blood flow values
had returned to baseline, subjects were administered 400 µg GTN as a sublingual spray and beat-to-beat blood pressure from the middle finger of the dominant hand was measured as described for visit 1.

5.3.4. Statistical analysis
All results are expressed as mean ± SD unless otherwise noted. All FBF values are presented as the ratio of the infused versus the non-infused arm (75,343). This approach normalizes the results obtained over time due to small alterations in sympathetic activation and/or blood pressure (471) and is considered more repeatable and reliable than absolute values of FBF in the infused arm alone (472). Normality was assessed for all variables in each of the three groups using the Shapiro-Wilk test. Several of the variables were not normally distributed; the data were transformed using the natural log, which yielded normal distribution. Within and between-group differences were evaluated with a repeated measures ANOVA. A doubly repeated-measures ANOVA was used to assess the differences in FBF between groups, the effect over time (co-infusions) as well as the group by time interaction. A value of $P<0.05$ was set as the threshold for significance. SAS software (version 9.2., SAS Institute Inc., Cary, North Carolina) was used for all statistical analyses.

5.4. Results

5.4.1. Heart rate and blood pressure responses
Results of heart rate and blood pressure responses are summarized in Table 5.1. Baseline standing heart rate and systolic blood pressure (SBP) did not differ significantly between the groups on visit 1. Heart rate increased significantly 3 hours after the first dose of transdermal GTN in GTN plus placebo and GTN plus atorvastatin groups and it remained significantly higher on visit 2 in the GTN plus atorvastatin group. After 6 days, heart rate decreased to baseline values in the GTN plus placebo group. Three hours after the administration of the first transdermal preparation, standing SBP was significantly lower in GTN plus placebo and GTN plus atorvastatin groups. On visit 2, SBP returned to baseline values in the GTN plus placebo group. In contrast, standing SBP in the GTN plus atorvastatin group remained lower when compared with baseline. There were no significant differences in blood pressure or heart rate at any time-point in the atorvastatin group. Blood pressure and heart rate did not change significantly in response to any of the intra-arterial drug infusions.
Table 5.1: Blood pressure and heart rate responses to transdermal GTN

<table>
<thead>
<tr>
<th></th>
<th>Visit 1</th>
<th>Visit 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>3hrs After GTN</td>
</tr>
<tr>
<td>Systolic Blood Pressure, mmHg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTN+Placebo</td>
<td>117±10</td>
<td>104±7*</td>
</tr>
<tr>
<td>GTN+Atorvastatin</td>
<td>116±10</td>
<td>103±8†</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>117±10</td>
<td>117±10</td>
</tr>
<tr>
<td>Heart Rate, bpm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTN+Placebo</td>
<td>79±12</td>
<td>100±15*</td>
</tr>
<tr>
<td>GTN+Atorvastatin</td>
<td>78±12</td>
<td>94±12†</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>74±12</td>
<td>77±13</td>
</tr>
</tbody>
</table>

* $P<0.001$ vs. baseline and visit 2

† $P<0.01$ vs. baseline and visit 2

‡ $P<0.05$ vs. baseline
5.4.2. Blood pressure responses to sublingual GTN administration

On visit 1, SBP responses to a single sublingual dose of 400 µg GTN were similar between groups (-11±3 mmHg, -9±3 mmHg, -10±4 mmHg for GTN plus placebo, GTN plus atorvastatin, and atorvastatin alone groups respectively, \( P=\text{NS} \)). On visit 2, SBP responses remained similar in the GTN plus atorvastatin and atorvastatin alone groups, and did not differ significantly from the values obtained on visit 1 (-9±3 mmHg, -9±4 mmHg for GTN plus atorvastatin, and atorvastatin groups, respectively; \( P=\text{NS} \)). In contrast, the reduction in SBP with sublingual GTN was significantly blunted in the GTN plus placebo group (-5±3 mmHg, \( P<0.001 \) vs. visit 1, \( P<0.05 \) vs. GTN plus atorvastatin and atorvastatin groups, Figure 5.1).

5.4.3. FBF responses

On visit 1, FBF was similar between the 3 groups (data not shown). On visit 2, when co-infused with saline, a dose-dependent increase in FBF in response to each infused concentration of ACh was observed in all groups. However, FBF responses were significantly blunted in the GTN plus placebo group compared with the GTN plus atorvastatin and the atorvastatin alone groups (\( P<0.01 \) for the effect of group; Table 5.2, Figure 5.2). When co-infused with vitamin C, ACh responses in the GTN plus placebo group were restored compared to saline confusion (\( P<0.01 \) for the interaction of time and group) and did not significantly differ from those in the GTN plus atorvastatin and atorvastatin groups (\( P=\text{NS} \) for the effect of group; Table 5.2, Figure 5.3). Vitamin C did not alter ACh-induced responses in the GTN plus atorvastatin or the atorvastatin alone groups.

5.4.4. Changes in lipid profiles

The changes in lipid profiles are summarized in Table 5.3. After 7 days of atorvastatin administration, significant decreases were observed in total cholesterol levels and LDL cholesterol in GTN plus atorvastatin and atorvastatin groups. There were no significant differences in lipid profiles between visits in the GTN plus placebo group nor were there any significant between-group differences.

5.5. Discussion

The present study demonstrates, for the first time in humans, the ability of the HMG-CoA reductase inhibitor atorvastatin to prevent the development of tolerance and endothelial
Figure 5.1: Blood pressure responses to sublingual GTN administration

Bar graph showing SBP responses to a single sublingual dose of 400 µg GTN expressed as the change in SBP from baseline. After chronic therapy with GTN, responses were significantly blunted in the GTN plus placebo group but were preserved in the GTN plus atorvastatin group and the atorvastatin alone group. Data are mean ± SEM.

* $P<0.001$ for within-group comparison vs. visit 1 † $P<0.05$ for between-group comparison vs. GTN plus placebo group.
### Table 5.2: FBF Responses

<table>
<thead>
<tr>
<th>Condition</th>
<th>GTN+Placebo</th>
<th>GTN+Atorvastatin</th>
<th>Atorvastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline/Saline</td>
<td>1.1±0.2 [0.1±0.2]</td>
<td>1.0±0.2 [0.0±0.1]</td>
<td>1.0±0.3 [-0.1±0.3]</td>
</tr>
<tr>
<td>Saline/ACh 7.5 µg/min</td>
<td>1.3±0.5 [0.2±0.4]</td>
<td>3.5±2.1 [1.1±0.6]</td>
<td>2.9±1.1 [1.0±0.3]</td>
</tr>
<tr>
<td>Saline/ACh 15 µg/min</td>
<td>2.2±1.3 [0.7±0.6]</td>
<td>4.6±2.5 [1.4±0.6]</td>
<td>4.1±2.0 [1.3±0.4]</td>
</tr>
<tr>
<td>Saline/ACh 30 µg/min</td>
<td>2.7±1.3 [0.9±0.5]</td>
<td>5.3±2.8 [1.5±0.5]</td>
<td>5.1±2.1 [1.6±0.4]</td>
</tr>
<tr>
<td>VitC/Saline</td>
<td>1.0±0.1 [0.0±0.1]</td>
<td>1.0±0.2 [0.0±0.2]</td>
<td>1.0±0.3 [0.0±0.3]</td>
</tr>
<tr>
<td>VitC/ACh 7.5 µg/min</td>
<td>2.9±1.3 [1.0±0.4]</td>
<td>2.8±1.3 [0.9±0.5]</td>
<td>3.1±0.7 [1.1±0.2]</td>
</tr>
<tr>
<td>VitC/ACh 15 µg/min</td>
<td>4.3±1.8 [1.4±0.4]</td>
<td>4.2±2.6 [1.2±0.7]</td>
<td>3.2±1.4 [1.1±0.4]</td>
</tr>
<tr>
<td>VitC/ACh 30 µg/min</td>
<td>6.1±2.9 [1.7±0.5]</td>
<td>4.8±2.7 [1.4±0.5]</td>
<td>4.9±2.5 [1.5±0.5]</td>
</tr>
</tbody>
</table>

Values are expressed as the ratio of the infused to the non-infused arms. Values in square brackets are log-transformed data.

* $P<0.01$ for ANOVA treatment effect between groups.
† $P<0.01$ for ANOVA group*time (co-infusion) interaction.

VitC, vitamin C.
Figure 5.2: FBF responses to ACh co-infused with saline

Responses to incremental intra-arterial infusions of ACh (7.5, 15, and 30 µg/min) co-infused with normal saline in the 3 groups. FBF is expressed as the ratio of infused to non-infused arm. FBF responses were significantly blunted in the GTN plus placebo group ($P<0.01$ for ANOVA treatment effect between groups). Data are mean ± SEM. Statistical analysis was performed after natural log transformation. NS, normal saline.
Figure 5.3: FBF responses to ACh co-infused with vitamin C

Responses to incremental intra-arterial infusions of ACh (7.5, 15, and 30 µg/min) co-infused with vitamin C in the 3 groups. FBF is expressed as the ratio of infused to non-infused arm. ACh responses were normalized in the GTN plus placebo group when co-infused with vitamin C (P=NS for ANOVA treatment effect between groups). ACh responses were significantly different when co-infused with vitamin C than during normal saline co-infusion (P<0.01 for ANOVA group by time interaction). Data are mean ± SEM. Statistical analysis was performed after natural log transformation. VitC, vitamin C; NS, normal saline.
### Table 5.3: Analysis of lipid parameters

<table>
<thead>
<tr>
<th></th>
<th>Visit 1</th>
<th></th>
<th></th>
<th>Visit 2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total Cholesterol</td>
<td>HDL</td>
<td>LDL</td>
<td>Triglycerides</td>
<td></td>
</tr>
<tr>
<td>GTN+Placebo</td>
<td>4.0±0.7</td>
<td>1.5±0.4</td>
<td>2.1±0.6</td>
<td>0.85±0.41</td>
<td>4.0±0.7</td>
<td>1.5±0.4</td>
</tr>
<tr>
<td>GTN+Atorvastatin</td>
<td>3.6±0.7</td>
<td>1.4±0.3</td>
<td>1.9±0.6</td>
<td>0.86±0.52</td>
<td>2.4±0.4*</td>
<td>1.3±0.2</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>3.8±0.7</td>
<td>1.5±0.3</td>
<td>1.9±0.5</td>
<td>1.01±0.84</td>
<td>3.0±1.2†</td>
<td>1.4±0.3</td>
</tr>
</tbody>
</table>

Data in mmol/L.

* $P<0.0001$ vs. corresponding value on visit 1.

† $P<0.05$ vs. corresponding value on visit 1.

HDL, high-density lipoprotein.
dysfunction associated with sustained GTN therapy in normal volunteers, an effect mediated, at least in part, by a reduction of oxidative stress in the vasculature.

Continuous treatment with GTN and other organic nitrates is associated with a loss of clinical efficacy and the development of important abnormalities in endothelial function. The mechanism behind these phenomena appears to be multifactorial including impaired biotransformation of GTN by mitochondrial aldehyde dehydrogenase, and possible neurohumoral activation (109). Furthermore, a large body of work points to the generation of ROS during sustained GTN therapy, initially from mitochondrial and eventually cytosolic sources, as a central component to this pathophysiology (206). Although the interactions of ROS are multiple, an important target is endothelium-derived NO, leading to the formation of peroxynitrite. Peroxynitrite can oxidize tetrahydrobiopterin, uncoupling the NOS enzyme and reducing endothelial NO bioavailability. In support of these hypotheses, previous reports have confirmed that therapy with GTN is associated with the development of significant endothelial dysfunction and NOS uncoupling in both the coronary and forearm circulation in humans (74,75), an effect that can be prevented by the coadministration of antioxidants (150).

The HMG-CoA reductase inhibitors have become standard therapy in patients with hypercholesterolemia and coronary artery disease and are effective in the primary and secondary prevention of cardiovascular events (344-350). It is now clear that the benefits associated with the HMG-CoA reductase inhibitors extend beyond cholesterol reduction, and that these drugs possess cholesterol-independent, or pleiotropic, effects (462). Such effects include antioxidant, anti-inflammatory, antithrombotic, as well as vascular-protective properties, possibly mediated by an increase in NO bioavailability. HMG-CoA reductase inhibitors have been hypothesized to increase NO bioavailability through multiple mechanisms. These include a direct increase in NOS enzymatic activity, through PI3K/ Akt-mediated phosphorylation; increased production of its essential cofactor tetrahydrobiopterin; and an increase in NOS mRNA half-life (376,462). Collectively, these pleiotropic effects may act both to improve and preserve vascular function in response to a number of risk factors or exposures that are associated with the development of endothelial dysfunction and atherosclerosis. Since treatment with organic nitrates, particularly GTN, is associated with the development of endothelial dysfunction, it has been hypothesized that HMG-CoA reductase inhibitors could modify the vascular responses to sustained nitrate therapy. This hypothesis was tested in 3 separate reports where atorvastatin, pravastatin, and
rosuvastatin administration were shown to prevent both endothelial dysfunction and nitrate tolerance in the arterial circulation of normcholesterolemic rats (440-442), at least in part, by preventing GTN-associated oxidative stress.

Findings from the current study suggest that atorvastatin prevents the development of nitrate tolerance in humans. On visit 1, standing blood pressure values were significantly lower than baseline values after 3 hours in those that received GTN. In contrast to the GTN plus placebo group, where blood pressure values returned to baseline after 7 days of continuous therapy, those in the GTN plus atorvastatin group had values that remained significantly lower than baseline indicating a sustained vasodilatory effect of GTN. In addition, blood pressure responses to sublingual GTN were significantly blunted in the GTN plus placebo group following 7 days of transdermal GTN therapy, an effect that was prevented by the co-administration of atorvastatin. These results are in agreement with the previously mentioned animal studies documenting a prevention of GTN tolerance through co-administration of an HMG-CoA reductase inhibitor (440-442).

Our results are in agreement with prior reports in animal models that atorvastatin prevents the development of endothelial dysfunction during continuous GTN therapy in humans. We found FBF responses to ACh in the GTN plus atorvastatin group to be significantly greater than those in the GTN plus placebo group after 7 days of treatment. In contrast, we observed a significant increase in FBF responses when ACh was co-infused with vitamin C in the GTN plus placebo group while there was no change in the GTN plus atorvastatin group. Taken together, these results suggest that an important component of atorvastatin’s ability to preserve endothelial responses is via a direct or indirect antioxidant mechanism. As mentioned above, the hypothesized sources of ROS in the setting of GTN-induced endothelial dysfunction are multiple, thus it is possible that atorvastatin may exert its antioxidant effects on multiple targets. Previous studies have suggested that HMG-CoA reductase inhibitors may counteract the GTN-induced increase in ROS by directly decreasing NADPH oxidase activity, by preventing NOS uncoupling and preserving NOS-mediated NO production, or both (440-442). Our group has previously demonstrated that the abnormal responses to ACh during chronic GTN therapy are at least partly related to abnormalities in NOS activity (75,343). This point was emphasized by the fact that these responses can be normalized by pharmacologically preventing NOS uncoupling (343). Similarly, chronic nitrate therapy has been demonstrated to cause an up-regulation in
endothelial NOS expression but decreased NO bioavailability due to uncoupling of the enzyme (157). Our observations suggest that HMG-CoA reductase inhibitors, in this case atorvastatin, have the capacity to restore NOS function via a mechanism which modifies the increased free radical response that occurs during sustained nitrate exposure (462). This, in turn, appears to prevent both the development of endothelial dysfunction and nitrate tolerance.

In the current study, the FBF responses to ACh in the GTN plus atorvastatin group were the same as those observed in the atorvastatin alone group. This raises the possibility that the effect of atorvastatin might depend on an independent potentiation of FBF responses, rather than a true prevention of nitrate-induced endothelial dysfunction. Two considerations suggest that this is not the case. First, the ACh-induced FBF responses in the atorvastatin alone group were very similar to those previously observed in our laboratory in healthy volunteers receiving no therapy (75), and previous reports consistently showed that HMG-CoA reductase inhibitors have a marginal (if any) impact on FBF responses to ACh in healthy volunteers (473,474). Second, the co-administration of vitamin C markedly improved the FBF responses in the GTN plus placebo group but did not change the responses in the GTN plus atorvastatin group, which suggests that atorvastatin and vitamin C act via similar mechanisms (i.e., by preventing GTN-induced oxidative stress).

5.5.1. Study Limitations
We observed significant reductions in both total cholesterol and LDL levels in subjects that received GTN plus atorvastatin or atorvastatin alone and thus we cannot discount that the lipid-lowering effect of atorvastatin may have contributed to the preservation of vascular function with chronic GTN. However, the above considerations suggest that our observations do not depend on simple potentiation of FBF responses induced by atorvastatin. We believe that these observations allow us to exclude the lipid-lowering effect of HMG-CoA reductase inhibitor administration as a potential explanation for our observations. The current study used a treatment period of 7 days of continuous therapy and the impact of longer periods of concurrent therapy, particularly in clinical practice, warrants further investigations. Importantly, endothelial dysfunction in patients with cardiovascular disease is also brought about by risk factors for cardiovascular disease such as age, smoking, hypertension, hyperlipidemia, and diabetes. For this mechanistic study, we elected to recruit normal volunteers to avoid the confounding effects of concurrent drug therapy that may also act to independently improve endothelial function. One
intriguing hypothesis raised by these findings is that almost all previous studies describing the phenomenon of nitrate tolerance and nitrate-induced endothelial dysfunction involved patients not receiving HMG-CoA reductase inhibitors. It is possible that the entire area of nitrate tolerance will require re-exploration in light of the potential effects of co-treatments such as vitamin supplements, HMG-CoA reductase inhibitors, angiotensin-converting enzyme inhibitors etc, which are now common treatment strategies in cardiovascular disease. Further studies will be needed in patients with overt atherosclerotic disease receiving treatment regimens currently being used in clinical practice.

5.6. **Conclusions**

We demonstrate the ability of atorvastatin to prevent the development of tolerance and endothelial dysfunction associated with chronic GTN therapy in healthy normal volunteers. Further, our data suggest that an antioxidant effect of atorvastatin is at least partly responsible in the prevention of these GTN–induced effects. We believe that our observations emphasize the need for more clinical investigations concerning the impact of HMG-CoA reductase inhibition on the efficacy and the vascular impact of organic nitrates.
Chapter 6 General Discussion

The objectives of the studies described in this thesis were firstly, to compare different methods of FMD measurement with regards to their repeatability, NO-dependency, and their sensitivity in distinguishing between healthy endothelial vasodilatory responses and impaired responses in individuals with cardiovascular risk factors and patients with cardiovascular disease. The second objective was to assess the impact of HMG-CoA reductase inhibitor therapy on the development of both conduit and resistance vessel vasodilatory impairment in the settings of IR injury and nitrate tolerance in humans in vivo.

6.1. Observations of time-based measures of flow-mediated dilation of forearm conduit arteries: Implications for the accurate assessment of endothelial function

While endothelial function is widely recognized as an important cause of cardiovascular disease, its assessment clinically and use as a surrogate marker of cardiovascular outcome have yet to be realized. Of the available methods to measure endothelial function, FMD likely holds the most promise, as it is a relatively simple, non-invasive measure of conduit arterial vasodilatory capacity. However, FMD suffers from methodological heterogeneity between laboratories, making it difficult to compare data between studies and laboratories. This may be due, at least in part, to a poor understanding of the physiological components and mechanisms behind the FMD response, most of which have yet to be elucidated.

6.1.1. Technical standardization of FMD

A good measure of vascular reactivity aimed at improving cardiovascular risk assessment, above having a sound physiological basis, should be reproducible, observer independent, and easily standardized (475). For the reasons mentioned above, the current measurement of FMD does not fit this description. If FMD is to become a high fidelity research tool and possibly a clinical tool, it is imperative that the “noise” due to technical heterogeneity be reduced (476). Radial and brachial artery diameters normally range in size from approximately 2 to 5 mm, such that a 1% increase equates to a 0.02 to 0.05 mm arterial dilation. Given the small “signal” produced by distal cuff occlusion, improper acquisition and/or analysis techniques can significantly compromise the validity of measurements.
6.1.1.1. The issue of cuff placement

With regards to variation in experimental setup during image acquisition, placement of the cuff used to induce the hyperemic stimulus has been one major source of methodological heterogeneity between laboratories (477). In the first reported FMD procedure, Celermajer et al. employed placement of the cuff distal to the ultrasound probe (80). However, many subsequent studies employed a proximal cuff placement as this elicits a greater peak hyperemic response and subsequent FMD in most cases (233,477-481). However, more recent studies have suggested that proximal cuff placement may recruit non-endothelial mechanisms due to a reduction in transmural pressure and the introduction of ischemia to the area of measurement (223). Proximal cuff placement has also been demonstrated to be less NO dependent, as it involves the contribution of EDHFs and prostaglandins (223,233). Interestingly, select studies also reported information on time to-FMD\text{max}, finding in all cases that it was longer with proximal cuff placement (478,480,481).

Importantly, evidence suggests that proximal cuff placement may be less sensitive to detect differences in responses between groups compared to distal placement. In a comparison of the two methods, distal cuff placement was shown to have superior repeatability when assessed using coefficients of variation, ICC, and Bland and Altman plots (478). Another study comparing FMD responses with distal and proximal cuff placement in cigarette smokers and age- and gender-matched healthy control subjects, it was found that FMD values were significantly lower in smokers using distal cuff placement (482). In contrast, no difference in FMD responses was noted between smokers and healthy controls when proximal cuff placement was employed. Further, the administration of the antioxidant allopurinol improved FMD responses with distal cuff placement in the smokers whereas these subjects did not demonstrate any change in their FMD responses with allopurinol when the cuff was placed proximally (482). Thus, the evidence currently available has led current published guidelines to recommend distal cuff occlusion for FMD experiments and has urged investigators to implement this technique (222,223).

However, a recent meta-analysis that included a total of 14 studies and 8300 individuals found that FMD performed with proximal occlusion was at least as predictive, if not more predictive, of future cardiovascular events as distal occlusion (483). While the authors acknowledged that larger studies should be performed to confirm these findings, they concluded that the predictive
value of FMD might not be solely related to its assumed NO-dependency. Nonetheless, the greater endothelial-dependency of distal cuff placement coupled with its greater repeatability have led to recommendations that the cuff be placed below the site of imaging.

6.1.1.2. Measurement of peak diameter following cuff deflation

As mentioned in section 1.2.1.5 of the introduction and in chapter 2, a second major area of technical variation is in the assessment of peak diameter following cuff deflation. The initial studies of Celermajer et al. employed the assessment of peak diameter on a single frame at 60 seconds post-deflation (80,246). Although recent papers have questioned the validity of this technique when compared to peak diameter assessment by continuous arterial diameter measurement after cuff deflation (272,273), this approach is still employed in many laboratories (223).

In the study described in chapter 2, we aimed to further compare these two methods of arterial diameter measurement; the traditional assessment that measures peak diameter at 60 seconds after cuff deflation (FMD$_{60}$) and continuous beat-to-beat assessment of arterial diameter after cuff deflation (FMD$_{max-cont}$); with regards to their repeatability, NO-dependency, and their sensitivity to distinguish healthy volunteers from those with compromised endothelial health.

The results described in chapter 2 demonstrate that continuous measurement of arterial diameter measurement likely improves on the traditional measurement at 60 seconds following cuff deflation as it is more repeatable and shows greater sensitivity in distinguishing between vasodilatory responses in healthy volunteers and smokers, hypertensives and patients with CAD, and HF. These results extend those of Black et al. who observed a 25-40% underestimation of the true FMD when calculated by FMD$_{60}$ (272). This led to decreased sensitivity in the ability to detect differences in the FMD response between young and older healthy volunteers. The decreased sensitivity of FMD$_{60}$ compared to FMD$_{max-cont}$ is in part, related to our observation that time to-FMD$_{max}$ shows a large degree of heterogeneity both within and between individuals, making it unrepeatable and unable to differentiate between healthy controls and those with compromised endothelial health. In agreement with Black et al., we did observe a significant relationship between time to-FMD$_{max}$ and age in healthy volunteers, an observation that might be related to a decrease in arterial compliance (275,484). As shown in Table 2.2, this decrease in sensitivity may lead to a reduction in the effect size of interventions and result in type II
statistical errors.

The variability associated with the measurement of FMD is the result of both technical and biological factors. While the mechanisms and basis of biological variability remain largely unexplored (discussed below), the contribution of technical variability, the easier of the two to control for, has still not been eliminated and continues to plague the utility of the measure. Our results are relevant to the improvement and eventual standardization of FMD methodology as they emphasize the importance of continuous diameter measurement throughout the FMD procedure. Indeed, a recent methodological guideline review, based on our observations and those of Black et al., has recommended the continuous measurement of arterial diameter of at least 180 seconds after cuff deflation (223).

6.1.2. Determining the mechanistic basis for FMD

With regards to the physiology of FMD, the response that governs a vasodilatory response upon release of an occluding cuff is a multi-step process that culminates in the dilation of the vessel. More specifically, the release of the occluding cuff initiates a shear stimulus that is sensed by mechanotransducing elements of the endothelial wall. These elements activate a signaling cascade that results in the production of vasoactive mediators, which then diffuse from the endothelial cell into the subadjacent smooth muscle cell. Once in the smooth muscle, vasodilators trigger another signaling cascade that results in a lowering of intracellular Ca\(^{2+}\) concentration and vasorelaxation. This vasorelaxation, coupled with the influence of structural proteins in the vessel wall such as collagen and elastin ultimately result in a change in arterial diameter that is measured with the ultrasound device. Following arterial dilation, the vasodilatory signal decays and diameter returns to baseline. It is possible that inter-individual heterogeneity may affect each step of this process, resulting in a different vasodilatory response to a given cuff occlusion (223). Further the precise mechanisms that govern each of these processes remain undiscovered.

With this in mind, we sought to investigate an additional component of the FMD response, namely time to peak dilation (time to-FMD\(_{\text{max}}\)), to determine whether the kinetics of the FMD response are NO-sensitive and whether they provide additional information to the traditional assessment of arterial diameter. Our results demonstrate that time to-FMD\(_{\text{max}}\) is not a NO-dependent variable and shows a large amount of heterogeneity both between and within
individuals. Further work will be needed to determine what governs the kinetics of the FMD response and the possible relationship between arterial compliance and time to $FMD_{max}$. Additionally, the utility of its measurement in the assessment of endothelial function remains to be determined.

Future studies will also need to be focused on determining the mechanistic basis for other components of the FMD response that contribute to the variability of the measure. Indeed, the measurement of FMD likely needs to be standardized to control for the multiple factors involved in the response if it is to become a clinical tool for assessing cardiovascular risk (485). In this regard, numerous groups have begun the attempt to normalize the FMD response by the shear stimulus, which is estimated by calculating shear rate. This is thought to account and control for the magnitude of the underlying stimulus that evokes FMD as well as reducing day-to-day variability associated with peripheral artery reactivity (222,223). More recent work in this area has led to the suggestion that Poiseuille’s law be used in the normalization procedure. Poiseuille’s law relates flow velocity (rate at which blood flows through the artery) with the radius of the vessel. In support of normalization, it has been reported that healthy older adults have a preserved endothelial function when FMD is normalized for post-deflation shear rates (486). In addition, it has been demonstrated that normalizing FMD for shear rate eliminates the influence of differing shear profiles created by differences in cuff occlusion duration (487). However, Poiseuille’s law is limited by the fact that it is not inherent to elastic, distensible arteries. Further, the non-Newtonian properties of blood in vivo and the fact that flow may not have a parabolic velocity profile in all vessels make Poiseuille’s law inapplicable to in vivo arterial dynamics (488,489). Additionally, the relationship between shear rate and FMD may be inconsistent depending on the cohort studied. Thijssen et al. (490) reported a weak correlation between shear and FMD with the poorest correlations observed in children and older adults. These results suggest that normalizing FMD for shear is age dependent and may only be appropriate when investigating young adults. Thus, although shear stress is clearly an important stimulus leading to artery dilation, the simple normalization by one factor appears inappropriate given the current limitations of Poiseuille’s law and potential differences in the shear rate-FMD relationship between cohorts. Nonetheless, the attempt of normalizing FMD to the shear stimulus is definitely a positive step and future studies addressing the biologic factors that contribute to the variability of FMD should definitely be encouraged.
It is important to emphasize that the FMD protocol employed in the study described in chapter 2 of this thesis involved measurements of the radial artery and may not represent responses in the larger, more commonly measured, brachial artery. The applicability of our findings to arterial diameter measurements from the brachial artery may not be as obvious as originally thought. Indeed, preliminary evidence has demonstrated a non-uniform response to a given shear stimulus in radial and brachial arteries (491). While the mechanism(s) for this heterogeneity in responses between the two vessels is currently unknown, it may be the result of physiological and anatomical differences between the vessels (491). Future studies will need to investigate this issue further.

6.1.3. Is FMD a clinical tool for assessing cardiovascular risk?

Given the above considerations, whether FMD can be a useful clinical measurement to assess cardiovascular risk in the future remains to be determined. While there is strong evidence that FMD independently predicts future cardiovascular events in patients with cardiovascular disease, the predictive value in asymptomatic patients is modest at best when current measurement protocols are employed. Risk assessment in asymptomatic patients, leading to the prediction of future cardiovascular events and targeting their prevention, should be considered one of the most important features of a clinical prognostic tool and therefore it is in these populations that a noninvasive technique like FMD would be most useful and cost-effective (211). Clearly the lack of protocol standardization remains a major drawback. However, current data regarding distal cuff positioning and continuous arterial diameter measurement are convincing and suggest that a standardized methodological protocol cannot be far from implementation.

In contrast, a significant amount of work may still need to be done to achieve an appropriate normalization of the FMD response to control for biological variation. Only then will the heterogeneity in the components of the FMD response between individuals be appropriately controlled and normalized in order to determine whether FMD is truly predictive in healthy individuals. Further, the fact that within-subject variability tends to be lower than the variability between-subjects possibly indicates that measuring the change in FMD over time may have better prognostic value than measuring FMD at a single time point. Indeed, a good measure of vascular reactivity should demonstrate an improvement in cardiovascular risk with an improvement in vascular reactivity (475). While this may indeed be the case in diseased patients (256,271,492), this has yet to be properly investigated in asymptomatic healthy individuals.
Another possibility that has recently been raised by Anderson et al. is that an impaired FMD may be an event that develops later on in the clinical progression of endothelial dysfunction and thus, may not have predictive value in otherwise healthy individuals. In 1574 healthy middle-aged men, it was found that hyperemic velocity and carotid intima media thickness, but not brachial artery FMD were predictive of future cardiovascular events over a mean follow-up period of 7 years (269). Further, the addition of hyperemic velocity to Framingham risk assessment resulted in a net clinical reclassification improvement of 28.7% after 5 years of follow-up in the cohort of subjects classified as being at intermediate risk for cardiovascular events. The authors hypothesized that an impaired hyperemic velocity, a measure of microvascular dysfunction, may precede that of the larger conduit vessels, thus making hyperemic velocity an earlier marker of endothelial dysfunction (269). Hyperemic velocity was previously demonstrated to correlate more strongly with traditional cardiovascular disease risk factors than FMD (249,493), and has been shown to have predictive value in patients undergoing non-cardiac vascular surgery (494). Another recent outcome study supports this hypothesis. Lind et al. found that resistance vessel responses to intra-arterial ACh, but not brachial artery FMD were associated with 5-year risk of future cardiovascular events in a population-based sample of Swedish seniors (219). Further studies are needed to test the validity of this hypothesis and to determine the clinical applicability of hyperemic velocity for the assessment of cardiovascular risk.

6.2. Cardioprotection with the HMG-CoA reductase inhibitors and the concept of sustained cardioprotection

The results described in chapter 3 demonstrated that single-dose rosuvastatin afforded significant endothelial protection against IR without changes in plasma lipids, suggestive that this effect may involve effects that are independent of lipid-lowering. This endothelial protection was lost when subjects were pretreated with the selective COX-2 inhibitor celecoxib, demonstrating the importance of the COX-2 enzyme in the cardioprotective-signaling cascade induced by rosuvastatin. These observations are in agreement with the evidence from animal models of IR injury suggesting marked myocardial and vascular protection with HMG-CoA reductase inhibitor pretreatment. Further, these observations provide a mechanistic explanation for the results of retrospective studies in patients undergoing percutaneous coronary interventions, coronary artery bypass grafting or experiencing acute coronary syndromes in which treatment
with HMG-CoA reductase inhibitors was consistently shown to reduce evidence of myocardial injury as well as cardiovascular morbidity and/or mortality (425-428,430-433,495). Similarly, five prospective randomized trials have demonstrated reduced incidence of myocardial injury, defined as proportion of patients with creatine kinase-MB, troponin-I, and myoglobin levels above the upper limits of normal, in patients receiving HMG-CoA reductase inhibitors before cardiac surgery or percutaneous revascularization (435,436,438,439,464). However, it remained unclear from these retrospective studies and prospective trials whether the protection offered by HMG-CoA reductase inhibitors was due to a direct preconditioning-like effect or to beneficial effects on coagulation, lipid status, and/or inflammation. Given our observation that rosuvastatin can prevent the significant impairment in endothelium-dependent vasodilation induced by IR, our data support the concept that, beyond their lipid-lowering effects, HMG-CoA reductase inhibitors act by triggering important cardioprotective effectors and mediators at the level of the endothelium.

Our mechanistic data provide further insight in these phenomena. Studies in animals suggest that the preconditioning-mimetic properties of HMG-CoA reductase inhibitors appear to be dependent upon an upregulation in NO production by NOS, as both eNOS and iNOS isoforms are upregulated after HMG-CoA reductase inhibitor treatment (384,402-408) and play a role in the subsequent downstream opening of the cardioprotective mK<sub>ATP</sub> channels (295,403). Other lines of evidence have demonstrated the importance of vasoactive prostaglandin production in the manifestation of this protective phenotype. Indeed, HMG-CoA reductase inhibitors have been shown to upregulate enzymes involved in the synthesis of prostaglandins: cytosolic phospholipase A<sub>2</sub>, COX-2, PGI<sub>2</sub> synthase, and prostaglandin E<sub>2</sub> synthase (410,411,414). Similarly, this upregulation of prostaglandin production has been shown to be both eNOS- and iNOS-dependent (411,413,414). These data suggest that pretreatment with HMG-CoA reductase inhibitors in humans might mimic, at least in part, the cascade triggered by IPC, as in vitro evidence suggests that this protective phenomenon is also associated with increased synthesis of COX-2 products such as prostaglandin E<sub>2</sub> and 6-keto-Prostaglandin F<sub>1α</sub> (303), and that administration of COX-2 inhibitors or high-dose aspirin prior to ischemia results in loss of protection (299,303). Similarly, opioid-induced preconditioning has been demonstrated to be both iNOS and COX-2-dependent (496). In line with this evidence, our study demonstrates that the endothelial protection afforded by rosuvastatin was abolished in the presence of COX-2
inhibition, suggesting that COX-2 is likely involved in rosuvastatin-mediated endothelial-protection in humans. To our knowledge, the current study represents the first demonstration of the importance of COX-2 in a human, in vivo model of cardioprotection.

Importantly, while COX-2 appears to play an important role in mediating cardioprotection, the enzyme COX-1 does not (410,411,413). Thus, our observations may have important clinical implications, as they may contribute, at least in part, to the clarification of the mechanism behind the observed increases in cardiovascular morbidity and mortality in patients receiving COX-2 inhibitors or non-selective non-steroidal anti-inflammatory agents such as diclofenac and ibuprofen (457,458,497-505). Under this hypothesis, such increases in morbidity and mortality may be the result of the inhibition of important cardioprotective signaling pathways activated in response to stressors such as ischemia, or the inhibition of pharmacologic agents that act upon important effectors and mediators of these same cardioprotective pathways (506).

6.2.1. Lack of direct cardioprotection with sustained therapy
The concept of creating chronic or sustained forms of cardioprotection stems from the limitations and drawbacks of classical acute preconditioning paradigms (507). Information regarding the therapeutic potential of sustained cardioprotection, however, and how to best exploit it remains relatively unknown. While countless strategies have been investigated in the setting of experimental IR with regards to their potential for limiting injury, none of these strategies have been adopted in generalized clinical practice (508). Results from clinical trials assessing the effectiveness of preconditioning strategies have been mixed and in the trials that have shown a benefit, the extent of tissue injury and functional recovery from IR has been much less than that observed in experimental protocols (509). While a large component of this translational failure is the result of poor experimental design (508,510), less attention has been given to the physiological limitations of the cardioprotective strategies being tested. For example, a large amount of data, including studies in humans, indicate that classical preconditioning strategies confer minimal protection in aged individuals (509,511-514), and in the presence of risk factors such as diabetes (515), hyperlipidemia (516,517), obesity (518), and hypertension (312). While not all studies have demonstrated a loss of cardioprotection by preconditioning strategies in these settings, collectively the data suggest that the threshold for cardioprotection may be increased, possibly due to impaired/desensitized cardioprotective signaling pathways (509,519). Further, therapeutic agents commonly administered in the coronary care setting, such as β-blockers and
non-steroidal anti-inflammatory drugs may interfere with preconditioning pathways and blunt the protective response (506,520). Our acute rosuvastatin and celecoxib data would support this hypothesis.

In contrast, preliminary studies of chronic or sustained forms of cardioprotection have demonstrated efficacy in aged and disease states and thus, may not suffer from the same physiological drawbacks as acute preconditioning modalities, described below (507). Thus, rather than acute preconditioning, the ability to maintain a “preconditioned state” over a prolonged period, through the use of remote preconditioning methods or through the administration of cardioprotective agents, may offer more potential as a clinical therapeutic strategy. Chronic or sustained preconditioning strategies offer another advantage over acute preconditioning methods in that they could also be applicable in the setting of acute MI.

The results from the study described in chapter 4 indicate that the protection against IR-induced endothelial dysfunction afforded by acute rosuvastatin administration is lost upon sustained therapy in humans. To date, evidence as to whether this benefit is maintained in patients who are on chronic HMG-CoA reductase inhibitor therapy remains controversial. Animal models of chronic therapy with these agents have involved the use of simvastatin, pravastatin, atorvastatin and rosuvastatin and have provided inconclusive results with respect to their effect on IR (415,416,420-422); while there are some reports of sustained protection (415,416,421), other studies have reported a loss of protection during sustained therapy (415,420,422,423). In humans, the Atorvastatin for Reduction of Myocardial Damage During Angioplasty-Recapture trial investigated whether acute atorvastatin reloading (80 mg 12 h before PCI) could protect patients receiving chronic statin therapy. In particular the study assessed the effect of atorvastatin reload on the 30-day incidence of major adverse cardiac events (cardiac death, myocardial infarction, or unplanned revascularization) (464). The results showed a reduction in 30-day incidence of cardiac events in patients on chronic HMG-CoA reductase inhibitor therapy who received the atorvastatin reload compared to patients receiving placebo, particularly in acute coronary syndrome patients. Although not tested directly, the significant reduction in the cardiovascular event rate compared to patients receiving atorvastatin reload vs. patients receiving placebo is suggestive of a waning of the cardioprotective benefit with chronic HMG-CoA reductase inhibitor therapy.
An important finding in the study by Mensah et al. was that an increase in the levels of the PI3K inhibitor PTEN was associated with chronic atorvastatin treatment. PI3K activates a signalling cascade, through Akt, that controls important cellular functions, including cell survival, growth and migration. Such functions are believed to play an important role in mediating the protective effects of acute IPC and acute pharmacologic preconditioning (465). More specifically, Akt may promote cell survival by phosphorylating and inhibiting proapoptotic proteins. These include glycogen synthase kinase-3-beta, Bad, and caspase 9 (465). At the same time, Akt can phosphorylate both antiapoptotic substrates, in order to promote cell survival. These include p70s6 kinase, eNOS, and the mouse double minute protein (465). Importantly, while the promotion of survival by this pathway may be of benefit in the acute setting, prolonged activation may be deleterious as it may promote hypertrophy and malignancy (521). As such, the body appears to maintain a balance between the acute activation and the harmful effects of sustained activation (521). How this is achieved is through the upregulation of PTEN, which is responsible for negatively regulating PI3K activation. Although regulation of PTEN expression remains poorly understood, acute ROS activation from NADPH oxidase or mitochondria may reversibly inactivate PTEN acutely and this may be the main process that acutely regulates its expression (465). Additionally, factors such as peroxisome proliferator-activated receptor gamma and the tumour suppressor p53 have been demonstrated to stimulate expression of the protein (465). Peroxisome proliferator-activated receptor gamma activity is increased with chronic HMG-CoA reductase inhibitor treatment (423,522) and thus, may contribute to any potential suppression of cardioprotection with chronic HMG-CoA reductase inhibitor administration, as in the current study. The impact of these mediators in HMG-CoA reductase inhibitor-mediated preconditioning, particularly in humans, remains unknown at present. Future studies will need to investigate these desensitization pathways in humans.

6.2.1.1. Sustained or chronic ischemic preconditioning

The concept of prolonged IPC has been explored in two recent reports using a swine model of IR injury. These studies tested the effect two different models of chronic stimulation, repetitive coronary stenosis and repetitive coronary occlusion, on myocardial infarct size after IR (466,467). Although a similar reduction in infarct size was observed with classic and sustained forms of IPC, it was demonstrated that these forms of IPC generate protection via different mechanisms. While classic IPC is highly dependent upon an upregulation in NO synthesis,
sustained preconditioning is not. Indeed, both iNOS gene expression and NOS activity were not different from control in swine that underwent sustained IPC (466). Additionally, the intravenous administration of the NOS inhibitor \(N\)-nitro-L-arginine before IR significantly increased infarct size in swine that underwent acute IPC compared to vehicle, but had no effect on final infarct size in those that underwent prolonged IPC (467). Further, the protection afforded by repeated IPC appears independent of PKC-\(\varepsilon\) activation and membrane translocation, another important mediator of classic preconditioning, as mentioned above. Using both microarray and quantitative PCR analyses, additional molecular pathways intrinsic to repetitive IPC and different from classical IPC were reported. The number of genes significantly regulated was greater in animals who received sustained IPC compared to classic IPC animals and of the 5739 genes regulated in sustained IPC, only 31\% were also regulated in classic IPC (467). Sustained IPC, but not classic IPC, showed downregulation of genes encoding proteins involved in mitochondrial oxidative metabolism and upregulation of genes involved in protein synthesis, unfolded protein response, autophagy, heat shock response, protein secretion, and an activation of the NF-\(\kappa\)B signaling pathway (467). While the results of these studies are encouraging and hypothesis generating, it must be noted that these chronic IPC protocols employed repetitive coronary stenosis or occlusion over a 96-hour period (i.e. a much shorter period than our 21-day sustained rosuvastatin protocol). At present, it is unknown whether such stimuli would demonstrate similar protection over a longer time period. Further, while the above studies argue against a role for iNOS in sustained IPC, others have documented a sustained cardioprotective response over a 2-month period following iNOS gene therapy (301). Thus, further studies are required to clarify the time-course and mechanistic basis of this unique window of cardioprotection.

6.2.1.2. Prolonged adenosine and opioid therapy

As mentioned in section 1.3.1.2, adenosine is considered an important trigger of the classical acute preconditioning response. The potential of chronically activating the adenosine \(A_1\) receptor has also been recently investigated in the hope of inducing sustained cardioprotection. Tsuchida et al. (523) compared 6 and 72-hour infusions of an adenosine \(A_1\) receptor agonist 2-chloro-N(6)-cyclopentyladenosine, and found that only the acute 6 hour infusion provided significant protection in reducing infarct size post-IR when compared to control. Interestingly, the authors also demonstrated that the initiation of IPC after the prolonged 72 h infusion failed to
elicit any protection, suggesting desensitization of the $A_1$ receptors or of the response. A subsequent study similarly documented refractoriness to traditional IPC after repeated stimulation of the adenosine $A_1$ receptor, corresponding to changes in release and activity of adenosine (524).

In contrast, a pronounced cardioprotective phenotype has been documented following 5-day exposure to morphine, suggesting that opioids may act as inducers of sustained cardioprotection (525). Importantly, sustained morphine exposure has been shown to confer a degree of protection that is significantly greater than that of acutely administered morphine in a mouse model of IR injury, and unlike acute preconditioning paradigms, this sustained protection is effective both in young and aged mouse hearts (526). Sustained morphine exposure has also been shown to confer protection from IR for up to 7 days after stimulus, a significantly longer than the window of protection than that created by acute preconditioning stimuli (527). Sustained opioid-induced protection against IR appears to be mediated by binding to the $\delta$-opioid receptor, and unlike acute preconditioning, seems independent of mK$_{ATP}$ channel activation, and is only partially dependent upon PI3K/Akt activation. Rather, sustained opioid protection appears to involve G protein-coupled receptor-mediated activation of protein kinase A, and $\beta_2$-adrenergic receptor-activation (527,528). However, the exact mechanism by which sustained opioid exposure acts to confer cardioprotection, and how this differs from acute exposure, remains poorly understood.

6.2.1.3. Prolonged GTN therapy
As with HMG-CoA reductase inhibitors, preconditioning responses to sustained exposure to GTN in animal models of IR have yielded conflicting results. While some studies have found sustained protective effects with GTN (315,529), others have documented that they are lost over time (530). In humans, only limited information is available concerning the prolonged pharmacologic preconditioning effects of GTN. Acute exposure to GTN has repeatedly been shown to have protective cardiovascular effects, both at the level of the endothelium (319) and the heart in patients with cardiovascular disease (316,317,452). In contrast, our laboratory has recently reported that repeated, daily 2-hour exposure to GTN over a 7-day period was associated with the loss of its acute preconditioning effects in forearm resistance vessels (339,531). The loss of protection was coupled to a reduction in heme-oxygenase-1 expression that is elevated upon acute GTN exposure and possesses cardioprotective properties (532).
For the moment, the available data suggest that any future study investigating the preconditioning effect of a physical or pharmacological intervention should also test whether this effect can be maintained over a prolonged period, as protection observed with acute administration does not necessarily assume the same protection with sustained administration. Future studies will also be needed to better understand the mechanism behind the sustained cardioprotective effect of repeated IPC, and how and where this mechanism diverges from the acute preconditioning signaling pathway. The lack of benefit with many pharmacologic strategies upon prolonged administration is suggestive of the fact that such agents are likely not targeting the effectors and mediators of this divergent signaling cascade. A better understanding of these signaling mechanisms will possibly allow for their pharmacologic manipulation. The possibility of prolonged preconditioning therapy that could protect at risk patients on an ongoing basis holds great promise. If pharmacologic agents with preconditioning properties can be shown to have sustained protective effects, without associated tachyphylaxis, there may be an opportunity to use such agents as a new therapeutic approach in high-risk patients.

6.2.2. Limitations of the experimental protocol

Limitations associated with the experimental model of IR in the human forearm need to be acknowledged. In contrast to animal models and clinical IR, it has been demonstrated that our forearm model of transient IR injury impairs endothelial vasodilatory responses while leaving smooth muscle responses unaltered (73). Thus, while we did observe a marked preservation of FMD following rosuvastatin administration, a direct cardioprotective effect of rosuvastatin on the underlying vascular smooth muscle cannot be inferred from these data. As such, the relevance of this finding to clinical IR, which is characterized by both vascular and tissue injury, is currently unknown. Similarly, the lack of endothelial protection observed with sustained rosuvastatin administration does not infer a similar loss of protection at the level of the smooth muscle. Further, while the impairment in FMD after IR is believed to be largely mediated by a dysfunctional endothelium (533,534), the contribution of other factors that may alter the FMD response (such as sympathoexcitation) in this model of IR remain poorly understood and require further investigation.
6.3. HMG-CoA reductase inhibitors in the setting of nitrate tolerance and nitrate-induced endothelial dysfunction

To date, there is little information regarding the effectiveness of HMG-CoA reductase inhibitors to prevent or reverse the development of tolerance and endothelial dysfunction associated with chronic nitrate therapy in humans. The results described in chapter 5 outline the ability of atorvastatin, when co-administered with continuous transdermal GTN, to prevent the blunting of FBF responses to ACh observed in those that received GTN alone. Additionally, atorvastatin coadministration preserved vascular responsiveness to sublingual GTN, suggestive of a prevention of tolerance development.

The study described in chapter 5 stemmed from 3 previously published reports investigating the effects of HMG-CoA reductase inhibitors on the prevention/reversibility of GTN tolerance and GTN-induced endothelial dysfunction in normocholesterolemic rats (440-442). The three reports involved a 5-week treatment protocol with pravastatin, atorvastatin or rosuvastatin, with subcutaneous injections of GTN (50 mg/kg/d) administered for the final 3 days of therapy. In all 3 reports, HMG-CoA reductase inhibitor pretreatment preserved the vasodilatory effect of GTN in excised thoracic aortic rings. This preservation of GTN activity was NO-dependent as the protective effect of the HMG-CoA reductase inhibitors on GTN-induced vasodilation was abolished following the administration of the eNOS inhibitor N-nitro-L-arginine methyl ester, and in eNOS knockout mice. However, unlike previous studies (453), HMG-CoA reductase inhibitor pretreatment did not alter eNOS protein expression, which led the authors to postulate that one mechanism whereby HMG-CoA reductase inhibitors counteract nitrate tolerance is by preventing uncoupling of NOS, rather than an increase in eNOS abundance (441,442). These 3 studies also provided evidence that HMG-CoA reductase inhibitor-induced reduction in NADPH-mediated superoxide forms another important mechanism for the prevention of GTN tolerance as they observed an acute loss of the vasodilatory response to GTN effect after the addition of NADPH to organ baths (440-442). Further, NADPH oxidase activity assays showed a significant increase in activity in rats receiving GTN alone, while enzyme activity was reduced back to control levels in those pretreated with HMG-CoA reductase inhibitors (441,442). HMG-CoA reductase inhibitor pretreatment also protected the endothelium from GTN-induced endothelial dysfunction as ACh-induced vasodilatory responses were normalized compared to rats that received GTN alone (441,442). Finally, the authors also demonstrated that the
protective effects of HMG-CoA reductase inhibitors were mediated by a cholesterol-independent mechanism as the addition of mevalonate abolished the protective effect on GTN vasodilatory responsiveness (442).

Our results extend these observations in a human model of nitrate tolerance and nitrate-induced endothelial dysfunction. Our laboratory has previously employed this model to demonstrate that 1-week of either continuous transdermal GTN therapy or once daily IS-5-MN administration significantly impairs FBF responses to ACh in otherwise healthy individuals (75,150). These blunted responses were associated with impairments in NOS function as the vasoconstriction and reduction in FBF elicited in control subjects by L-NMMA was significantly blunted in subjects treated with GTN (75,343). This NOS impairment may be related to an increase in ROS production as the intra-arterial co-administration of vitamin C with ACh completely normalized responses in subjects receiving GTN (150,339). Importantly, coadministration of high-dose folic acid, which is hypothesized to restore eNOS function by increasing intracellular BH₄ levels (535) and acting as a mild antioxidant (536), can also completely prevent the impairment resistance vessel vasodilatory responses associated with continuous GTN administration as well as restore the vasoconstrictive response to L-NMMA (343). The current results outline a similar ability of high-dose atorvastatin to prevent the GTN-induced blunting of FBF increases to ACh when co-administered with continuous transdermal GTN over 1 week. Further, the intra-arterial co-administration of vitamin C with ACh restored FBF responses in individuals who received GTN and placebo while there was no effect in those that received GTN plus atorvastatin. This observation suggests that atorvastatin and vitamin C may act through a similar anti-oxidant mechanism to preserve forearm ACh responses in this setting. Our evidence showing sustained systolic blood pressure reductions with transdermal GTN as well as a preserved response to a sublingual GTN challenge further suggest a prevention of GTN tolerance development with atorvastatin coadministration. Lastly, although between-group comparisons for GTN plus atorvastatin and atorvastatin groups did not reach statistical significance, our finding of what appeared to be a greater LDL reduction with atorvastatin is interesting. There is currently no evidence of a positive interaction between statins and organic nitrates with regards to LDL lowering, and this would be an interesting hypothesis. On the other hand, the small sample size employed in the current study may have accounted for the greater reduction in plasma lipids observed in the GTN plus atorvastatin group compared to atorvastatin alone, and no definitive
conclusions can be drawn.

Mechanistically, it remains unknown how HMG-CoA reductase inhibitors prevent tolerance development. The animal studies described above provided evidence to suggest that a lipid-independent prevention of NOS uncoupling contributed to the observed responses. Further, these same studies and our current study in healthy humans indicate that a potent antioxidant effect may play a role in the prevention of GTN tolerance. It is possible that HMG-CoA reductase inhibitors may act at other cellular sites, yet uninvestigated, also contribute to prevent tolerance development. One such site proposed by others is the mitochondria, which plays a crucial role in the initial development of GTN tolerance, as mentioned in section 1.1.4.4 (109). GTN has been hypothesized to cause mitochondrial uncoupling and the premature release of partially reduced oxygen from mitochondrial complex I or III (205,206), depolarization of mitochondrial membrane potential, and mitochondrial swelling (207). Previous studies provide evidence that HMG-CoA reductase inhibitors can play a role in regulating mitochondrial function. In one such study, hydrogen peroxide-induced depolarization of the mitochondrial membrane potential was prevented in isolated neonatal cardiac myocytes that were pretreated with simvastatin 1-hour prior (537). This preventative effect was absent in the presence of the eNOS inhibitor N-nitro-L-arginine methyl ester leading the authors to hypothesize that simvastatin acted to acutely augment NO bioavailability, likely by Akt phosphorylation, and subsequently prevent excessive mitochondrial membrane depolarization. Such an effect would prevent excessive mitochondrial dysfunction, superoxide generation and apoptosis (537). It is likely that HMG-CoA reductase inhibitors exert similar mitochondrial regulatory effects in the vasculature (403), however, future studies will be needed to elucidate the precise mechanism and the various targets of the HMG-CoA reductase inhibitors in the setting of nitrate tolerance.

It is acknowledged that the study of healthy volunteers, while allowing us to assess the interaction of HMG-CoA reductase inhibitors and GTN during continuous administration in the absence of risk factors and concurrent drug therapy, does limit the clinical relevance of our findings. However, we believe our data are clinically relevant and hypothesis generating. Whether similar results would be observed in patients receiving current treatment regimens is unknown and clinically important. Our laboratory has previously demonstrated that continuous transdermal GTN administration for 5 days augments the constrictive response to ACh in the coronary circulation of CAD patients (74). However, the majority of these patients were not
receiving ACE inhibitors or anti-oxidants, and none of the patients enrolled were treated with HMG-CoA reductase inhibitors. If it is determined that the co-administration of HMG CoA-reductase inhibitors does prevent tolerance to clinical end-points (such as treadmill-walking times) then it may be that the current assumptions about the development, the mechanisms and the implications of nitrate tolerance need to be reconsidered, in particular the need for intermittent dosing regimens, which may also introduce harm. Further studies will need to address this important clinical issue.

To date, a number of studies have suggested that antioxidants can prevent the development of nitrate tolerance; the majority of these studies were carried out in normal volunteers (340,342,469) while one report was performed in patients with chronic heart failure (341). Importantly, in patients with chronic CAD and angina it does appear that the efficacy of nitrates needs to be revisited as most of the evidence regarding the development of nitrate tolerance was developed before the generalized introduction of HMG CoA-reductase inhibitors, but also the newer 3rd generation β-blockers and renin-angiotensin active agents, all of which have theoretical interactions with the development of nitrate-induced tolerance. Additionally, mechanistic observations such as the current study are typically short-term in nature and thus, it is not possible to determine whether the effects of atorvastatin are maintained with longer administration periods. As such, future studies should also examine the impact of longer treatment periods on tolerance development.

Whether atorvastatin coadministration would similarly prevent tolerance with other nitrates is unknown based on the present data. Nitrate tolerance remains a complex multifactorial phenomenon and many of these factors remain incompletely understood. As mentioned in section 1.1.4.4, while the evidence for the current mitochondrial-oxidative stress hypothesis of nitrate tolerance is strong, it appears incompatible with tolerance development with isosorbide dinitrate and IS-5-MN that are not biotransformed in the mitochondria. Further, the existence of cross-tolerance with GTN, NO donors and these agents are similarly not explained by the inactivation of mALDH-2 (141,147-149). Although antioxidant therapy has been shown to reverse endothelial dysfunction associated with sustained IS-5-MN therapy (150), the potential mechanistic differences between GTN tolerance and tolerance with other organic nitrates make it impossible to preclude a similar effect with atorvastatin. Adding further complexity to the issue is the suggestion that the mechanism of tolerance development may differ between different
vascular beds. Future studies will be needed to determine whether atorvastatin similarly prevents tolerance in different vascular beds, such as in veins.

6.4. Summary of experiments and conclusions

Experimental work in this thesis began with the assessment of different methods to define the dilatory response of the conduit artery to distal wrist cuff occlusion during the FMD procedure. We observed that the continuous measurement of arterial diameter is superior to its measurement at a fixed time point (in this case 60 seconds). Specifically, the continuous measurement of arterial diameter was found to be repeatable and more sensitive in distinguishing healthy subjects from those with compromised endothelial responses. As such, the measurement of continuous arterial diameter may provide a larger effect size for interventions and reduce the risk of type II statistical errors. These observations were, at least in part, related to the fact that the time to maximal arterial dilation after cuff deflation was highly heterogeneous and unrepeatable, both within individuals and between groups of healthy volunteers and those with risk factors or disease. Mechanistically, we observed that while both measures of arterial diameter were highly NO dependent, the time to peak dilation was NO independent.

The remaining studies were focused on assessing the endothelial-protective effects of 2 HMG-CoA reductase inhibitors, rosuvastatin and atorvastatin. The first of these studies employed the FMD technique, calculated using continuous arterial diameter measurement, in the assessment of conduit artery, endothelium-dependent, vasodilation as a measure of endothelial function before and after local IR injury. Specifically, we investigated the effect and mechanistic basis of rosuvastatin-induced protection from IR-induced endothelial dysfunction in the forearm of normal healthy volunteers. We hypothesized that rosuvastatin would induce a preconditioning-like effect on the endothelium and that this effect would be due to the cholesterol-independent, vascular-protective properties of this agent. Acute rosuvastatin administration was associated with a preserved radial artery FMD response after 15 minutes of ischemia and 15 minutes of reperfusion in comparison to subjects who received placebo. This effect was observed without changes in plasma lipids, suggestive that it was mediated by properties that are independent of cholesterol reduction. We then aimed to determine whether the vascular-protective benefit afforded by rosuvastatin was dependent upon the presence of the COX-2 enzyme, similar to what has previously been demonstrated in animal models of IR. We found that pretreatment with a
selective COX-2 inhibitor resulted in the abolishment of rosuvastatin’s protective benefit on the vasodilatory response following IR. These observations provided the first demonstration in humans of the ability of rosuvastatin to pharmacologically prevent the development of IR-induced endothelial dysfunction. Further, our data demonstrate the importance of COX-2 in the manifestation of this cardioprotective phenotype, which may provide potential mechanistic insight into the observed cardiovascular toxicity with selective COX-2 inhibitors and nonselective non-steroidal anti-inflammatory drugs.

We then assessed whether rosuvastatin afforded similar endothelial protection against IR upon sustained administration. The fact that most, if not all, patients are receiving chronic HMG-CoA reductase inhibitor therapy make it necessary to determine whether sustained administration provides similar protection to acute administration and whether this protection is also COX-2 dependent. Further, the drawbacks of acute cardioprotective strategies prevent their translation in the clinical setting, and warrant the investigation into methods of sustained preconditioning that appear uninfluenced by these same drawbacks. We hypothesized that prolonged rosuvastatin administration at a clinically relevant dose would be associated with a preserved protective effect against IR-induced endothelial dysfunction. In contrast, we observed a loss of endothelial protection against IR after 21 days of rosuvastatin administration. Thus, while the chronic administration of HMG-CoA reductase inhibitors is clearly effective in the primary and secondary prevention of cardiovascular events, this appears to occur despite our current evidence suggesting the loss of direct cardioprotective effects. Of importance, these data support the concept that cardioprotection conferred following the acute administration of a pharmacologic agent does not guarantee similar protection upon chronic administration, and that sustained preconditioning strategies are likely through different cardioprotective signaling pathways. These findings have generated important future research objectives, namely to determine the mechanistic discrepancies between acute pharmacologic cardioprotection and sustained administration as well as the mechanistic differences between repeated IPC and pharmacologic strategies.

In the final study of this thesis, we demonstrated the ability of atorvastatin co-administration to prevent the development of tolerance and endothelial dysfunction associated with chronic nitrate therapy in healthy volunteers. This observation is of significance in that it questions the current assumptions about the development, the mechanisms, and the implications of nitrate tolerance.
Future studies will need to confirm our observation in patients with CAD and angina using clinically relevant endpoints and longer durations of therapy. Given the widespread use of vascular-protective agents such as HMG-CoA reductase inhibitors, angiotensin converting enzyme inhibitors, angiotensin receptor blockers, and 3rd generation β-blockers, it may be that the questions and concerns regarding the efficacy of organic nitrates will need to be re-investigated.
Appendices

Appendix 1 Measurement of endothelial function by venous-occlusion strain gauge plethysmography

1.1. Principles of the technique

The venous-occlusion plethysmography technique, first described over 100 years ago, represents one of the oldest methods to assess blood flow in humans *in vivo* (538). The underlying principle of the technique has remained the same since its introduction. cuffs placed on the upper-arm are inflated to a pressure that prevents venous drainage of the forearm but leaves arterial inflow uninterrupted. In this scenario, forearm volume increases linearly and is proportional to arterial flow. This relationship between forearm volume and arterial inflow holds true only if the forearm veins are not fully distended. At the point when venous pressure rises above that of the occluding cuff, the relationship is lost, permitting blood to escape the forearm compartment (471). In order to prevent this the upper arm occluding cuffs are inflated for a 10 second period and then deflated to permit venous drainage. After a 5-10 second deflation period, measurements can be repeated. In addition, blood flow to the hands is excluded by inflating wrist cuffs to suprasystolic pressures as the hand circulation contains a large amount of arterio-venous shunts and is more sensitive to temperature, which causes blood flow to be non-linear (471,539).

The method is based on the principle that changes in forearm volume can be deduced from changes in forearm circumference, assuming that forearm length remains the same and that the increase in circumference upon venous-occlusion occurs evenly along the length of the forearm. The increase in forearm volume that occurs with upper-arm cuff inflation is measured with the plethysmograph by mercury-in-silastic strain gauges. The strain gauges act as resistors; as forearm volume increases there is a corresponding increase in arm circumference that increases the length of the strain gauge. This is detected by the strain gauge as a change in electrical resistance and therefore a change in electrical potential. The recording is calibrated by inputting a selected percentage change in voltage (1% in our laboratory), which corresponds to the same percentage change in strain gauge length. Therefore, the percent change in strain gauge length per unit time (recorded in seconds) can be derived from the deflection of the strain gauge voltage recording from baseline over the 10-second inflation period. This is directly proportional to the
rate of change in limb volume or arterial inflow and is expressed in ml/100ml of forearm tissue/minute.

The study of FBF responses is commonly performed at baseline and following the intra-brachial infusion of vasoactive agents such as ACh, bradykinin, substance P, or NOS inhibitors. The intra-arterial administration of these agents permits the use of subsystemic doses, thus permitting the study of local drug effects. In particular, the intra-arterial infusion of ACh is thought to induce a local vasodilatory response primarily by stimulating the production and release of NO (2,212,540). Importantly, limitations must be noted when interpreting vascular responses to ACh. First and foremost, forearm ACh responses are commonly used as a surrogate for coronary endothelial function. However, while endothelial dysfunction in the coronary circulation is recognized as a paradoxical vasoconstriction (47), the same does not occur in the forearm where endothelial dysfunction is classified as blunted ACh responses compared to control (539). Additionally, in the presence of the nonspecific NOS inhibitor L-NMMA, ACh responses are only partly inhibited (75,541,542), indicating that ACh responses are partly NO independent.

1.2. Methods
All plethysmography studies are performed in the morning in a quiet, temperature and humidity-controlled room. Study subjects are required to refrain from any form of exercise for at least a 12-hour period prior to the study. Subjects are asked to comfortably lay supine on a bed. Prior to the commencement of the study, the brachial artery of the non-dominant arm is cannulated under local anesthetic (1% lidocaine) using a 20-gauge plastic catheter (Cook, Bloomington IN). Once cannulated, the catheter is connected to a manifold that includes luer connections for attachment of infusion tubing as well as a pressure transducer for acquisition and monitoring of invasive brachial artery blood pressure. The manifold is also attached to a 0.9% normal saline bag that is wrapped in a pressure administration cuff and inflated to 300 mmHg. A fast-flush valve is used to maintain a continuous infusion rate of 3 mL/hr in order to prevent thrombosis around the catheter.

Following arterial cannulation, both forearms are positioned above the level of the heart to allow for sufficient venous drainage during FBF measurement. Both upper-arms are supported with foam pads such that subjects are not elevating their arms under their own power. Cuffs are placed at the level of the upper-arms and wrists, as mentioned above (Upper arm: Hokanson 13 x
Upper-arm cuffs are connected to a rapid cuff inflator (Hokanson), while wrist cuffs are connected to a manual sphygmomanometer. Upper-arm and wrist cuffs are connected to their respective inflation device with Y air hoses such that the cuffs on each arm are inflated simultaneously. Strain gauges (Hokanson mercury-in-silastic strain gauges) are placed on the widest part of both forearms and are connected to the plethysmograph (Hokanson). Once all equipment has been attached, subjects are allowed to rest quietly for a period of at least 20 minutes.

Prior to beginning the measurement period, the wrist cuffs are inflated to suprasystolic pressure for 1 minute and baseline recordings are performed to confirm measurement stability. The plethysmograph is then balanced and calibrated using a 1% calibration signal. Measurements are performed upon rapid inflation of the upper-arm cuffs to 40 mmHg for 10 seconds. The cuffs are then deflated for 10 seconds prior to beginning the subsequent measurement. FBF is calculated as the mean of 5 consecutive measurements. Signals from the plethysmograph are outputted to an amplifier coupled to an ink recorder as well as to a digital converter and are, thus, acquired in hard copy as well as digitally on a laboratory computer.

1.3. Methodological considerations

FBF responses are measured on both the infused and non-infused forearm, as described above, and are expressed as a ratio of the FBF response in the infused/non-infused arm. This approach normalizes the results obtained for the normal variability of FBF over time due to small systemic alterations such as sympathetic activation, mood, temperature, and changes in blood pressure (471,472). This approach has also demonstrated superior repeatability and reliability when compared to absolute values of FBF in the infused arm alone (472). It is therefore recommended that responses to intra-arterial infusions should be measured using bilateral forearm plethysmography with the results expressed as FBF ratios. Coefficients of variation from unpublished repeatability data in our laboratory were between 15 and 20% for both FBF ratios and absolute FBF values.
Appendix 2 Measurement of endothelial function by flow-mediated dilation

2.1. Methods

All FMD studies are performed in a quiet, temperature and humidity-controlled room. Study subjects are required to refrain from any form of exercise for at least a 12-hour period prior to the study. Study subjects are asked to rest for at least 20 minutes in the supine position before measurements are started. Radial artery images are acquired with a GE Vivid7 (GE Healthcare, Mississauga, Ontario) instrument using a 14-MHz matrix array linear transducer. A probe holder is used during all studies to enable stable transducer position. End-diastolic, ECG-gated, longitudinal, B-mode images of the artery 10-15 cm below the antecubital fossa are digitally acquired and stored for off-line analysis. All studies are simultaneously recorded on VHS as a backup. Baseline diameter is acquired for at least 1 minute before cuff inflation. Subsequently, a pneumatic cuff placed at the level of the wrist (i.e., distal to the site of radial artery measurement) is inflated to 250 mm Hg for 4 minutes, 30 seconds. Arterial diameter is recorded continuously during baseline diameter assessment, during the period of cuff inflation, and for another 4 minutes and 30 seconds after wrist cuff deflation. Semi-automatic custom-designed software is used to calculate the arterial diameter from the trailing edge to the leading edge of the intima-blood interface (455). The software system has been validated for accuracy, reproducibility, and repeatability (455). FMD is calculated as the maximum percent increase in arterial diameter in the measurement period following cuff deflation, as compared to resting diameter. Baseline and hyperemic radial artery blood flows are calculated by multiplying time-averaged mean flow velocity of 3 cardiac cycles, acquired using pulsed-wave spectral Doppler at an insonation angle of 60°, by the vessel cross-sectional area.
**Appendix 3 Forearm model of IR injury**

The human *in vivo* forearm model of IR-induced endothelial dysfunction was developed and first described by Kharbanda et al. in 2001 (73). The protocol involved inflating a 12-cm-wide cuff at the level of the brachial artery to a pressure of 200 mm Hg for 20 minutes followed by 15 minutes of reperfusion. Conduit artery endothelial responses were measured by FMD at baseline, 15, and 60 minutes after reperfusion. Resistance vessels responses to increasing doses of intra-arterial ACh were assessed by forearm venous-occlusion strain gauge plethysmography at baseline, 15, 30, and 60 minutes following reperfusion. Endothelium-independent responses to sublingual and intra-arterial GTN were also assessed in both conduit and resistance vessels respectively. The authors found radial artery FMD to be significantly reduced at 15 minutes of reperfusion while the response returned to baseline at 60 minutes of reperfusion. Similarly, FBF responses to ACh were significantly reduced compared to baseline when measured each at 15, and 30 minutes of reperfusion. In contrast to conduit vessel responses, FBF responses to ACh remained significantly blunted at 60 minutes of reperfusion.

FBF responses to GTN were unchanged compared to baseline at 15 and 30 minutes of reperfusion, with a slight reduction observed at 60 minutes, while no change in radial artery dilation to GTN was observed at 15 and 60 min of reperfusion. Investigators also measured systemic levels of the neutrophil adhesion molecule CD11b and platelet neutrophil complexes in both arms and found that both plasma markers were significantly increased at 15 minutes of reperfusion compared to baseline in the control arm. No change was observed in the ischemic arm. The authors hypothesized that the lack of plasma marker elevation in the ischemia arm may have been due to sequestration of activated cells in the ischemic arm that were likely adhered to the vascular endothelium. This study demonstrated that the transient impairments in neutrophil adhesiveness and endothelial responses associated with IR in both conduit and resistance vessels could be prevented by IPC (three cycles of 5-minute upper arm cuff inflation followed by 5 minutes of reperfusion). It is important to note that others have not been able to demonstrate the same benefit of IPC in the human forearm (543).

A follow-up study by the same group investigated a potential mechanism responsible for the protective effect of IPC in the forearm. Broadhead et al. (294) demonstrated that the protective effect of IPC in forearm resistance vessels could be inhibited with the intra-arterial infusion of
the mK\textsubscript{ATP} channel blocker glibenclamide. Similar to animal models of IPC, this study emphasized the important role of the mK\textsubscript{ATP} channel in the manifestation of IPC-mediated protection. Similarly, the mK\textsubscript{ATP} channel opener diazoxide was shown to mimic the protection afforded by IPC, effects that were lost with the coadministration of glibenclamide. More recently, this same group demonstrated the importance of NADPH oxidase-mediated ROS production in the development of endothelial dysfunction in the forearm IR model. They were able to show that patients with chronic granulomatous disease, characterized by a molecular lesion in a subunit of NADPH oxidase that renders the enzyme inactive, are innately protected against IR-induced endothelial dysfunction in forearm conduit vessels (544).

Other groups have employed this model to demonstrate that pharmacologic agents shown to induce a preconditioning effect in animal models induce a similar effect in the human endothelium. Pernow et al. (533) demonstrated that a 15 minute-intra-arterial infusion of L-arginine after 15 min of ischemia significantly preserved FBF responses to ACh compared to NS or D-arginine infusions. Protection against IR in the forearm has also been demonstrated following intra-arterial infusions of bradykinin (534), vitamin C (545), the oral administration of the endothelin-1 antagonist bosentan (546), as well as the inhalation of sevoflurane (547) in forearm resistance vessels.

### 3.1. Data from our group

Our laboratory also has considerable experience with the forearm IR technique. Gori et al. (318) demonstrated that the phosphodiesterase-5 inhibitor sildenafil administered 2 hours prior to IR could completely prevent the blunting of radial artery FMD after 15 minutes of ischemia and 15 minutes of reperfusion. Similar to the study by Broadhead et al. with IPC, the protective effect of sildenafil was blocked when subjects were pre-treated with the K\textsubscript{ATP} channel blocker glibenclamide. In contrast, the B-vitamin folic acid (10mg/day for 7 days), which has been hypothesized to preserve NO production by NOS in settings of reduced BH\textsubscript{4} levels, did not preserve radial artery FMD values following IR (444).

Our group has also had an interest in the preconditioning properties of the organic nitrates on the human vascular endothelium. GTN (transdermal 0.6 mg/hr) administered for a 2 hour period 24 hours prior to IR was demonstrated to significantly attenuate the blunting of FMD with IR (319). This protective effect was dependent upon the generation of ROS and the transient opening of
the mPTP as both the co-administration of intravenous vitamin C and the pretreatment with the mPTP inhibitor cyclosporine blocked GTN-induced protection. A subsequent study investigated the preconditioning properties of other organic nitrates and demonstrated that pentaerithrityl tetranitrate was also able to preserve conduit artery FMD responses after IR while IS-5-MN pretreatment provided no protection (445). In contrast to GTN, vitamin C had no effect on pentaerithrityl tetranitrate-mediated preconditioning. It is possible that the induced expression of heme oxygenase and ferritin observed when this agent was incubated with isolated human endothelial cells, two genes whose products have been implicated as key players in both ischemic and pharmacologic signaling cascades, was involved in mediating the endothelial protective effect. More recently, we investigated whether daily 2-hour exposure to GTN maintains the preconditioning effects observed with acute administration. Unlike the acute setting, daily GTN exposure was unable to prevent the blunting of FBF responses to ACh following IR, demonstrating a loss of its preconditioning effects upon prolonged administration (339).

Finally, methodological data from our group are suggestive of a differential susceptibility to ischemic damage in large arteries compared to the microcirculation. Cutaneous reactive hyperemia and ACh-induced microvascular vasodilation measured by laser Doppler iontophoresis was used to assess the effect of IR on the skin microvasculature (548). While, radial artery FMD was significantly blunted after IR as shown previously, no effect was observed on microvascular reactive hyperemia and ACh-induced vasodilation.

3.2. **Postconditioning and remote preconditioning**

Investigators have additionally demonstrated that the forearm IR model is responsive to other forms of preconditioning, namely postconditioning and remote preconditioning. In the first of such studies, Kharbanda et al. (549) investigated the effects of remote preconditioning (three cycles of 5-min upper arm cuff inflation/deflation on the contralateral arm) on FBF responses to ACh following IR. Similar to classic IPC, remote preconditioning prior to IR prevented the attenuation in ACh responses after 15 minutes of reperfusion observed in the control arm. Loukogeorgakis et al. (550) subsequently followed this previous observation by investigating the time-course and potential mechanism behind remote preconditioning in the human forearm. The investigators found that remote preconditioning (three cycles of 5-min upper arm cuff inflation/deflation on the contralateral arm) preserved FMD responses after IR when performed.
immediately before IR, at 24 and 48 hours before IR but not when performed 4 hours before IR. Additionally, infusion of the nicotinic receptor antagonist trimetaphan, which blocks autonomic ganglia and therefore blocks both sympathetic and parasympathetic activity, during R-IPC attenuated early and late protection afforded by remote preconditioning. In the absence of remote preconditioning, trimetaphan had no effect on baseline FMD or on the blunting of FMD post-IR.

This same group also demonstrated that postconditioning (reperfusion interrupted by 3-4 cycles of short (10-30 seconds) periods of ischemia) similarly exerts potent endothelial protection in the forearm model of IR measured in the conduit artery by FMD (551) and also when the postconditioning episodes were performed remotely in the contralateral arm or leg, termed remote postconditioning (552). Importantly, not only do these studies demonstrate the effectiveness of postconditioning strategies in the forearm model of IR, but they also support the notion that at least part of the endothelial damage that occurs during experimental IR occurs during reperfusion.

3.3. **Forearm IR model in patients with cardiovascular disease**

The above examples outline the use of the forearm model of IR in healthy volunteers. Although the use of this model in patients has been limited to date, there is evidence to suggest that the model is also effective in studying endothelial damage and protection from IR in the forearm vasculature of diseased patients. Endothelial function of the radial artery assessed by FMD was measured in six patients with CAD was found to be significantly blunted following IR. Importantly, this impairment in endothelial function was prevented by remote preconditioning (552). Furthermore, patients with peripheral artery disease subjected to 20 minutes of forearm ischemia exhibited significantly reduced FBF responses to ACh at 15 and 60 minutes of reperfusion compared to baseline. This impairment in resistance vessel endothelial function was reversed with intra-arterial infusion of vitamin C (545).
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