Assessment of endothelial function in humans: Observations concerning the use of low flow-mediated constriction and the vascular effects of Nebivolol

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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Abstract

We first examined the effect of inflammation-induced by injection of Salmonella typhi vaccine on endothelial function. This was performed using flow-mediated dilation (FMD) combined with low flow-mediated constriction (FMC). We believe that combining these two endpoints would increase the sensitivity and specificity of endothelial function assessment. Unfortunately, after completion of the study, manufacturer of the vaccine announced that several batches of its typhoid fever vaccine, including the batch used in this study, had been recalled due to concerns of inadequate antigenicity.

We then investigated the ability of the third generation beta-blocker, nebivolol, to protect against endothelial dysfunction associated with ischemia and reperfusion (IR) injury. Using the FMD technique, we demonstrated that the acute administration of nebivolol has a pharmacologic preconditioning effect, providing protection against IR-induced endothelial dysfunction. We also proposed to examine the ability of nebivolol to protect against nitrate tolerance and nitrate-induced endothelial dysfunction. Unfortunately, this proposal could not be completed due to shortage of time.
Acknowledgement

The success and final outcome of this project would have not been possible without guidance and assistance from many people and I am extremely fortunate to have got this all along the completion of my project work. Everything I have done is only due to such guidance and assistance and I would like to thank them.

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<th>Full Form</th>
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<tbody>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ALDH2</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BH₄</td>
<td>Tetrahydrobiopterin</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>EDHF</td>
<td>Endothelium-derived hyperpolarizing factor</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>FBF</td>
<td>Forearm blood flow</td>
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<tr>
<td>FMC</td>
<td>Flow-mediated constriction</td>
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<tr>
<td>FMD</td>
<td>Flow-mediated dilation</td>
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<tr>
<td>GTN</td>
<td>Nitroglycerin</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IPC</td>
<td>Ischemic preconditioning</td>
</tr>
<tr>
<td>IR</td>
<td>Ischemia-reperfusion</td>
</tr>
<tr>
<td>IRI</td>
<td>Ischemia-reperfusion injury</td>
</tr>
<tr>
<td>mPTP</td>
<td>Mitochondrial permeability transition pore</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>sGC</td>
<td>Soluble guanylyl cyclase</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
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Introduction

1.1. Normal functions of the endothelium

A single layer of endothelial cells that lines the luminal vessel wall separates the blood from interstitial space and the vascular smooth muscle.\textsuperscript{1,2} Although the endothelium forms a single layer of cells, its total volume is comparable to that of liver.\textsuperscript{2} For decades, the endothelium was thought to only act as a passive barrier to the diffusion of macromolecules from the lumen to the interstitial space. Over the past 30 years, additional roles of endothelium have been defined and it is now believed that endothelium is involved in communication between blood and surrounding tissues.\textsuperscript{1,3} The endothelium plays a key role in maintaining vascular homeostasis and is believed to be a “storehouse of biologically active substances”.\textsuperscript{3} Endothelial cells are metabolically active components of vascular wall\textsuperscript{4} that control vascular tone through a balance in the production of vasodilators and vasoconstrictors.

The integrity of endothelial cells is very important in circulatory function and vessel wall maintenance.\textsuperscript{5} One of the major communication pathways of the endothelium is the L-arginine-nitric oxide-cGMP pathway. This cascade begins with nitric oxide (NO), which is generated from L-arginine and molecular oxygen by the enzyme endothelial nitric oxide synthase (NOS). NO is a free radical that controls basic biological functions.\textsuperscript{1} Although this is well known today, the association between endothelium-derived relaxing factor (EDRF) and NO is a relatively new biologic discovery, first described in the late 20\textsuperscript{th} century; until that time NO was primarily believed to be a toxic environmental pollutant found in cigarette smoke and other harmful gases. As a free radical, gaseous, inorganic, and uncharged diatomic molecule makes NO unique as a second messenger.\textsuperscript{6} Endothelial NOS, which produces NO, is stimulated in response to physicochemical stimuli such as increased in shear stress and receptor-dependent agonists such as acetylcholine (ACh) and bradykinin.\textsuperscript{1} NO is able to diffuse easily between the cells and tissues to react with a range of molecules.\textsuperscript{1,6} Diffusion of NO into the adjacent smooth muscle results in its interaction with soluble guanylate cyclase (sGC). This interaction leads to an increase in cGMP production that causes hyperpolarization of smooth muscle cells, inhibition of calcium influx with subsequent vasodilation.\textsuperscript{1} Basal vascular tone of large arteries is largely determined by blood flow which stimulates the release of endothelium-derived NO. Among the vasoactive substances released by the endothelium, NO has evoked much interest as it is considered to be the most potent endogenous vasodilator released in the body. Furthermore, the biologic activity of NO is a key marker of endothelial function and dysfunction.\textsuperscript{6,7} NO also acts in an autocrine fashion to prevent adhesion of leukocyte to the vessel wall either by interfering with the ability of the
leukocyte adhesion molecule CD11/CD18 to form adhesive bond with endothelial surface or by decreasing the expression of CD11/CD18 on leukocytes. Furthermore, it has been demonstrated that NO inhibits DNA synthesis, mitogenesis, and proliferation of smooth muscle cells.\(^8\) NO is able to diffuse into the cytosol of platelets, activate platelet sGC, and enhance production of cGMP. This leads to a reduction in platelet activation, as well as inhibition of platelet aggregation and adhesion.\(^9\) Inhibition of platelet aggregation and adhesion by NO results in protection of smooth muscle from exposure to platelet-derived growth factors. Therefore, NO has inhibitory effects on many phenomena involved in atherogenesis. Based on the effects mentioned above, NO is felt to be critical anti-atherogenic mechanism in the vasculature.\(^8\)

There are three isoforms of NOS in body named for the tissues in which they were first cloned; neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2), and endothelial NOS (eNOS, NOS3). Each NOS isoform is expressed in a variety of tissues and cell types.\(^10,11\) NOS enzymes are heme-containing enzymes that catalyze oxidation of L-arginine to NO and citrulline. Only 50-55% sequence identity exists between the three isoforms. However, high sequence identity exists in certain regions of the proteins, mainly in domains containing binding sites for tetrahydrobiopterin (BH4), heme, and calmodulin.\(^10\) In general, nNOS and eNOS are considered as constitutive, calcium-dependant enzymes which continuously produce low levels of NO.\(^6,10\) NO synthesized from these two isoforms are involved in intracellular signalling.\(^10,11\) Mice lacking eNOS manifest endothelial dysfunction, are hypertensive and demonstrate a more severe response to vascular injury, cerebral ischemia, and diet-induced atherosclerosis. Mice lacking nNOS demonstrate a less severe outcome in response to cerebral ischemia but increased diet-induced atherosclerosis.\(^12\) Since iNOS is calcium-independent and regulated by cytokines, it is believed to be the enzyme that is stimulated during immune response and NO produced by this isoform is involved in inflammatory processes.\(^10,11\) Reduced hypotension in the setting of septic shock have been observed in mice lacking iNOS.\(^6,12\) Of the three isoforms, eNOS is believed to be the major isoform responsible for NO production under physiological conditions in the cardiovascular system.\(^6\) All three isoforms share the same enzymatic mechanism, which involves the oxidation of L-arginine at the site of terminal guanidine nitrogen. They all require a number of cofactors for proper function, such as BH4 and nicotinamide-adenine-dinucleotide phosphate (NADPH).\(^12\) Functions of all the three isoforms of NOS are summarized in figure 1.1. Dimerization of NOS is necessary for enzymatic activity. Each monomer of NOS consists of a N-terminal oxygenase domain, which contains binding sites for BH4, molecular oxygen, and L-arginine. Each also contains a prosthetic heme group, and a C-terminal reductase domain that contains binding sites for flavin.
mononucleotide (FMN), flavin adenine dinucleotide (FAD), and reduced NADPH. Formation of NO by NOS requires two steps; the first step involves hydroxylation of L-arginine to N-hydroxy-L-arginine which stays bound to the enzyme; the second step involves oxidation of N-hydroxy-L-arginine to L-citrulline and NO.\textsuperscript{8,13} All NOS isozymes catalyze the electron transfer from NADPH on the C-terminal to the heme on the N-terminal. During NO synthesis, electrons are transferred from NADPH to the heme center located in the oxygenase domain via flavins. The electrons are used to reduce and activate O\textsubscript{2} for synthesis of NO.\textsuperscript{8}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{image}
\caption{Nitric oxide synthase isoforms and activities.}
\end{figure}

The endothelium releases various vasoactive substances other than NO such as prostaglandins, endothelium-derived hyperpolarizing factor (EDHF), and endothelin. These substances are released as a result of both humoral and mechanical stimuli and can intensely affect both the structure and function of vascular smooth muscle cells.\textsuperscript{5} Prostaglandin-I\textsubscript{2} or prostacyclin (PGI\textsubscript{2}) is derived from metabolism of arachidonic acid by cyclooxygenase (COX). PGI\textsubscript{2} has a short half-life and is fairly unstable. It activates adenylyl cyclase (AC) with subsequent increase in formation of cyclic adenosine monophosphate (cAMP) resulting in blood vessel relaxation.\textsuperscript{9} PGI\textsubscript{2} has limited role in regulation of vascular tone in humans. However, plays an important role in regulation of platelet function.\textsuperscript{14}
As mentioned above, the endothelium also releases EDHF to control vascular smooth muscle cell relaxation. EDHF refers to a number of vasodilator chemicals (or physical forces) that exert their action by activating different families of potassium channels in the adjacent vascular smooth muscle, hyperpolarizing the cell membrane, resulting in vascular smooth muscle relaxation. To date, a variety of arachidonic acid metabolites from the COX enzymes, lipooxygenases (LOXs), cytochrome P450 dependent pathways, hydrogen peroxide, carbon monoxide, and hydrogen sulfide have been identified as EDHFs. The list of EDHF’s is not complete and other compounds may be added in future. Studies have shown that the EDHF pathway appears to play minor role in regulation of normal vascular homeostasis but is believed to play a more important role in the setting of vascular disease and reduced activity of the NO pathway.\textsuperscript{15}

Three types of endothelin are produced in the body with only endothelin-1 (ET-1) being formed in vascular endothelial cells.\textsuperscript{5} ET-1 is released in response to shear stress and is involved in blood flow regulation.\textsuperscript{16} Vascular effects of endothelin are exerted through endothelin receptors, of which three types have been identified; ET\textsubscript{A}, ET\textsubscript{B}, and ET\textsubscript{C}. The specificity of the three receptors vary for the three endothelin peptides and activate different signaling pathways. ET\textsubscript{A} is predominantly present in vascular smooth muscle while ET\textsubscript{B} is abundant in endothelial cells.\textsuperscript{17} ET-1 stimulates cell proliferation and activates ET\textsubscript{A} receptors in vascular smooth muscle causing vasoconstriction. In the setting of normal physiology, the NO and ET systems are important, balancing determinants of vascular tone; ET-1 decreases the vasodilator effect of NO and NO in turn reduces vasoconstriction induced by ET-1.\textsuperscript{16} In situations where NO bioavailability is impaired, the pressor effect of ET-1 may be unmasked contributing to increased basal vascular tone and heightened sensitivity to vasoconstriction.

Endothelial and smooth muscle cells express a number of proteins which are directly involved in haemostasis. Expression of tissue factor results in activation of factor X which combines with factor Va. This converts prothrombin to thrombin. Thrombin is a multifunctional protein with both anticoagulant and pro-coagulant effects. Thrombomodulin, which is expressed on endothelial cell surface, binds to thrombin. Binding of thrombomodulin blocks binding of fibrinogen, platelet, and factor V to thrombin because they bind to the same site on thrombin. Therefore, all of these functions are blocked and thus inhibits coagulation.\textsuperscript{5}

In summary, the endothelium plays a major role in vascular function by production of a wide range of paracrine substances (figure 1.2). These substances, released by endothelium, play major roles in regulating vascular tone and permeability, inflammatory responses, thrombogenicity, as well as...
angiogenesis. The endothelium is an important organ, maintaining vascular homeostasis by promoting equilibrium between vasoconstriction and vasodilatation, inhibition versus promotion of proliferation and migration of smooth muscle cells, stimulation and inhibition of platelet activity and the maintenance of a balance between thrombogenesis and anticoagulant forces. Any sort of abnormality in this tightly balance results in a state of dysfunction.²,¹⁵

Figure 1.2. Functions of the endothelium. Endothelial cells control cellular function in the body through formation of a vast variety of mediators.
This figure is modified from Br J Anaesth 2004;93:105-13.

1.2. Pathophysiology of endothelial dysfunction

1.2.1. Oxidative stress and endothelial dysfunction:
Cardiovascular risk factors initiate a chronic inflammatory process that is accompanied by an increase in vasoconstrictor and prothrombic products. The endothelium releases leukocyte chemotactic factors, adhesion molecules, and inflammatory cytokines when exposed to a variety of stimuli. Furthermore, abnormalities in endothelial function are associated with increased risk of cardiovascular events, likely because the abnormal endothelium is associated with an increased risk of thrombosis and a greater tendency to vasoconstriction. Excess production and/or bioavailability of reactive oxygen species (ROS) is believed to be the fundamental abnormality that leads to altered vasoreactivity in the setting of endothelial dysfunction. ROS present an unpaired electron, making
them highly reactive with the various cellular components. The phrase ‘endothelial dysfunction’ refers to a wide range of changes in endothelial phenotype that are associated with abnormalities in function and appear to be atherosclerosis and to plaque formation and rupture. This term was established in the mid-eighties by Furchgott and Zawadzki who discovered that in the absence of the endothelium, exposed to ACh caused paradoxical constriction of vascular smooth muscle. Although many different diseases are associated with endothelial dysfunction, oxidative stress is felt to be a common denominator. Importantly, oxidative stress can result from either a decrease in the production of NO or from an increase in ROS, both of which create what is best termed a decrease in NO bioavailability. Of note, a change in NO bioavailability is most often results from a combination of decreased NO production in the face of concurrent increased ROS activity. Two of the most biologically important ROS in cardiovascular system are superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂). There are several important sources of vascular ROS production including NADPH oxidase, xanthine oxidase, lipooxygenase, cytochrome P450, uncoupling of mitochondrial respiratory chain, and the uncoupling of eNOS. The multiple sources of increased free radical production can be triggered by exposure to inflammatory cytokines, growth factors, disturbed flow conditions, and in response to stimuli such as angiotensin II (Ang II). Antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidise, thioredoxins, and peroxiredoxins control levels of ROS. ROS play an important role in vascular physiology and pathophysiology. Although low levels of ROS are necessary for vascular function, chronic excessive production or impaired function of antioxidant systems, coupled with a proinflammatory environment, can lead to produce marked abnormalities in vascular function. Such vascular oxidative stress, which is manifest by abnormalities in endothelial function, is accompanied by monocyte adhesion, platelet aggregation, inflammatory gene induction, vascular smooth muscle cell apoptosis, proliferation, and migration, matrix degradation, and impaired endothelium-dependent relaxation. Increased expression of adhesion molecules attracts monocytes, which transmigrate and form macrophages which in turn produce larger amounts of ROS. ROS can alter the balance between endothelial proliferation and apoptosis and can lead to either excessive angiogenesis or loss of endothelial cells. Interaction of NO and O₂⁻ leads to inactivation of NO, a decrease in its vasodilatory and anti-inflammatory effects, along with generation of peroxynitrite (ONOO⁻). This latter is a potent oxidant capable of oxidizing sulfhydryl groups, hydroxylation and nitration of amino acids such as tyrosine, tryptophan, and guanine, with subsequent injurious effects on vascular cells. In addition, peroxynitrite has potent oxidant effects on BH₄ resulting in eNOS uncoupling, reduced NO production and a subsequent increase in the production of O₂⁻ (detailed below), all of which lead to impaired vasodilation. In addition, peroxynitrite inhibits guanylyl cyclase,
inactive prostacyclin synthase, and inhibits SOD compounding both the loss of vasodilation and the
decrease in ROS bioavailability. Furthermore, chronic action of $O_2^-$ decreases the activity of calcium-
activated potassium channels involved in EDHF-mediated responses. In addition, ROS facilitate the
mobilization of calcium and increase the sensibility of contractile proteins to calcium ions promoting
the contraction of vascular smooth muscle cells.\textsuperscript{2} Endothelial dysfunction is the characteristic feature
of atherosclerosis progression and studies have documented that it is a significant independent
predictor of cardiovascular event rates.\textsuperscript{1} Endothelial dysfunction is not only the primary etiology of
atherosclerosis, but is also the earliest identifiable event in atherosclerosis process.\textsuperscript{7}

**Figure 1.3.** Sources and roles of ROS in response to injury. ROS produced in endothelial cells (EC) and vascular smooth muscle
cells (VSM) impair vessel tone and promote an inflammatory response and increase smooth muscle cell migration,
proliferation, and apoptosis. LOX: Lipooxygenase; CyP450: Cytochrome P450.

One of the fundamental abnormalities within the endothelium, which leads to altered vasoreactivity
and dysfunction, is a decrease in NO bioavailability due to uncoupling of NOS. Electron transfer within
NOS is tightly regulated and any disturbance leads to dissociation of ferrous-dioxygen complex and
generation of $O_2^-$ instead of NO. This switch from NO synthesis to $O_2^-$ production by NOS is referred to
as NOS uncoupling. The uncoupling of NOS leads to an amplification of the problem of reduced NO
bioavailability whereby increased free radical stress produces a greater tendency for NOS to uncouple
with subsequent decreases in NO production and increases in production of $O_2^-$, NOS uncoupling
occurs when concentration of L-arginine and/or BH$_4$ is lower than optimal.\textsuperscript{8} The enhanced generation
of $O_2^-$ leads to formation of peroxynitrite\textsuperscript{2} which consumes bioavailable NO, further enhances $O_2^-$
production by oxidation of zinc cluster within NOS, and dissociation of NOS dimer, as well as oxidizing BH₄. Thus, although eNOS facilitates maintaining endothelial NO-mediated quiescent state under normal conditions, under certain circumstances, it generates ROS initiating a vicious circle of ROS production and activation.

Endothelial dysfunction has been associated with hypertension, atherosclerosis, heart failure, acute myocardial infarction, renal failure, coronary syndrome, angina, thrombosis, intravascular coagulation, microalbuminuria, dialysis, Type I and Type II diabetes, hypercholesterolemia, smoking, obesity and a host of other clinical conditions associated with increased rates of adverse cardiovascular events. Mounting evidence suggest endothelial dysfunction is an important pathophysiological link between cardiovascular risk factors and development of atherosclerosis. The presence of endothelial dysfunction serves as an early marker of atherosclerosis and is considered as a valuable prognostic tool in predicting both the development of atherosclerosis and subsequent adverse clinical events. The progression of endothelial activation and dysfunction to atherosclerosis is complex and multifactorial but multiple lines of evidence suggest that abnormalities in NO bioavailability and consequent endothelial dysfunction play a key role in its development. In addition, many other pathophysiological processes such as abnormal responses to ischemia-reperfusion (IR) injury have been linked to endothelial dysfunction.
Figure 1.4. Potential mechanism by which cardiovascular risk factors lead to endothelial dysfunction. Many vascular diseases upregulate the NADPH oxidase and eNOS in parallel. Their respective products rapidly react and form ONOO\(^{-}\). This oxidizes BH\(_{4}\) and produces oxidative damage to eNOS. Therefore, reduction of O\(_{2}\) by eNOS is uncoupled from NO production and NOS switches its function from a NO-synthesizing enzyme into a \(\mathrm{O}_2\)-synthesizing enzyme and contribute to vascular oxidative stress.

1.2.2. Inflammation:

Previous studies have shown that there is an association between infection/inflammation and the risk of cardiovascular diseases. This association exists in two patterns. First, there is an association between chronic low-grade inflammation/infection and the slow process of atherogenesis. Second, there is an association between acute systemic inflammation and a transient increase in the risk of acute cardiovascular events.\(^{80}\) Although chronic inflammation associated with specific chronic diseases, such as connective tissue disorders, has been associated with accelerated atherosclerosis, it has become clear that chronic inflammation is associated with the atherosclerotic process even in the absence of a specific inflammatory disease state. This has been highlighted by the fact that nonspecific markers of inflammation, such as C-reactive protein (CRP), are potent predictors of both vascular disease and future cardiac events. From this perspective, it is now recognized that abnormalities in endothelial function appear to be mediated, at least in part, by inflammatory mediators and that such
inflammation represents a mechanistic role, along with other more traditional risk factors, in the development of atherosclerosis. The endothelial dysfunction associated with chronic inflammation occurs through a variety of mechanisms such as increased vascular oxidative stress, activation of redox-sensitive transcriptional pathways, and decreased eNOS function. Enhanced formation of reactive oxygen species in vascular wall and reduced endothelial function have all been associated with systemic inflammation. Therefore, while inflammation per se appears to play a causal role in atherosclerosis under certain specific circumstances (such as a chronic, systemic inflammatory disorder) vascular inflammation appears to play a role in the pathogenesis of atherosclerosis in general.

1.3. The effect of ischemia-reperfusion injury

1.3.1. On myocardial function:
Perfusion of an organ with oxygenated blood is necessary for cellular viability, end-organ function, and survival. Timely coronary reperfusion is the most efficient way to limit infarct size after acute myocardial infarction. As such, early and successful restoration of blood flow to the ischemic area is the principle objective in the treatment of patients in the setting of myocardial infarction. However, ironically, reperfusion itself is associated with an increase in downstream tissue damage, a phenomenon referred to as ischemia and reperfusion injury. Reestablishment of blood flow and oxygen supply causes a dramatic increase in free radical production, is associated with mechanical injury from the reperfusion pressure head and abnormal contractile performance as contractile function is restored despite the presence of local acidosis and marked abnormalities of membrane function and calcium kinetics. The injury resulted from restoration of blood flow to an ischemic tissue is defined as ischemia-reperfusion injury (IRI) and is a phenomenon of direct clinical relevance.

IR injury is a highly complex and multi-faceted phenomenon. Despite the ability of reperfusion to limit infarct size, a critical determinant of clinical outcome, reperfusion itself is associated with reperfusion-induced arrhythmias, myocardial stunning, and cell death. IR injury involves interaction between the vascular endothelium, interstitial components, circulating cells, and numerous other biological entities. The ischemic period leads to several changes that promote cell injury. A decrease in oxidative phosphorylation results in ATP depletion, calcium homeostasis derangement, and subsequent parenchymal damage through tissue necrosis. The most important sub-cellular effectors of IR injury are believed to be ROS. The production of free radicals in the myocardium increases exponentially during ischemia. Upon reperfusion, after an ischemic period, a further burst of ROS generation occurs, generated primarily from the mitochondrial respiratory chain complex. The ROS
produced cause cellular impairment in a number of ways such as membrane lipid peroxidation, enzyme denaturation, and intracellular organelle dysfunction. Free radical production is accompanied by excess production of hypoxanthine which cannot be broken down due to inactivity of oxygen-dependent xanthine oxidases. Upon reperfusion, the accumulated hypoxanthine has the potential for activation into ROS by-products, such as superoxide anion and hydroxyl radicals, which have the ability to interact with most cellular components. In addition, calcium influx into the mitochondria is enhanced in response to ischemia-reperfusion. This occurs, in part, by ROS mediated calcium release from the sarcoplasmic reticulum. The resulting increase in calcium leads to mitochondrial calcium overload, cellular hypercontracture, mitochondrial swelling, and finally cell death. The mitochondrial permeability transition pore (mPTP) is a non-selective channel in the inner mitochondrial membrane, was first proposed to be involved in IRI in 1987. Studies have shown that the mPTP remains closed during ischemia and only to open during the reperfusion. During reperfusion, further influx of calcium into mitochondria, a burst of oxidative stress, and rapid correction of acidosis occur. All of these factors increase the probability of mPTP opening in the first few minutes of reperfusion. Opening of mPTP leads to solute and water influx, and mitochondrial matrix swelling, ROS and proapoptotic mediators exit. More importantly, it results in collapse of mitochondrial membrane potential and further uncoupling of oxidative phosphorylation, eventually leading to ATP depletion and cell death. During ischemia, acidification of the intracellular space occurs due to anaerobic glycolysis and hydrolysis of high-energy phosphates. During prolonged ischemia, mitochondrial electron movement eventually ceases, which leads to disruption of mitochondrial proton-motive force that normally pumps protons into the mitochondrial matrix to create electrochemical gradient required for ATP synthesis, resulting in impaired ATP production. Continued use of ATP in the absence of production results in significant decrease in ATP concentrations. During ischemia, protons accumulate and lead to a rapid decrease in interstitial and intracellular pH. Upon reperfusion, the Na+/H+ exchanger is activated to correct the low intracellular pH and results in massive Na+ influx. High levels of intracellular Na+ stimulates sarcolemmal Na+/Ca2+ exchanger and leads to intracellular Ca2+ overload and contributes to lethal reperfusion injury. Importantly, ROS can deactivate NO and lead to an imbalance during IRI in favor of ROS which results in direct harm on local tissue and reduces NO and its protective effects.
1.3.2. On endothelial function:
In comparison to cardiomyocytes, endothelial cells are highly resistant to ischemic injury. Nevertheless, the endothelium is susceptible to injury during IR injury. A study by Quillen JE et al. demonstrated that ischemia without reperfusion causes only mild abnormalities in endothelium-mediated vascular responses while ischemia followed by reperfusion causes marked abnormalities in endothelium-mediated relaxation. These observations suggest that endothelium-dependent dysfunction is an important consequence of reperfusion injury. Multiple mechanisms appear to be involved in the development of endothelial dysfunction in response to IR-induced tissue injury; uncoupling of eNOS plays a central role in this phenomenon and leads to reduction in NO bioavailability which in turn results in significant impairment of endothelium-dependent vasodilation. Uncoupling of eNOS is also associated with enhanced production of ROS via activation of both NADPH oxidase and xanthine oxidase, principal sources of ROS. In addition, IR injury is associated with endothelial inflammatory response which is characterized by leukocyte adhesion and neutrophil activation. Endothelial cells are susceptible to apoptosis following IR. Apoptosis is an active, genetically programmed, energy consuming mechanism of cell death. TNF-α production is believed to play a crucial role by activation of caspases. But TNF-α is not the only cause and mitochondrial cytochrome c release following mPTP formation seems to be involved as well.

1.4. Nitrate tolerance and nitrate-induced endothelial dysfunction

1.4.1. Pharmacology of nitroglycerin and other organic nitrates:
Nitroglycerin (GTN) and other organic nitrates are important drugs, commonly prescribed for patients with coronary artery disease and congestive heart failure. GTN and other organic nitrates induce vasorelaxation through a similar mechanism as endogenous NO. Organic nitrates are prodrugs that release NO (or some NO moiety) after a biotransformation process that involves denitration of the nitrate. NO activates sGC, elevates tissue levels of the second messenger cGMP, reduces intracellular calcium, and leads to vasorelaxation. The clinical effects of GTN and other organic nitrates are mediated primarily by dilatation of capacitance veins as well as large conductance arteries while much higher concentration are required to dilate arterioles. Dilatation of conductance veins reduces cardiac volume and preload while dilatation of conductance arteries in combination with reduced left ventricular volume leads to a decrease in afterload. The reduction in preload and afterload by nitrates results in decreased cardiac work and lowers myocardial oxygen requirements. Nitroglycerin may also improve blood flow to the areas of ischemia by dilation of stenotic conduit (epicardial) coronary segments and/or by dilatation of coronary collateral vessels. In addition to their vascular effects,
nitrates have been demonstrated to inhibit platelet aggregation and adhesion, although the clinical relevance of such effects has never been determined.\textsuperscript{42}

Organic nitrates are metabolized by pathways that are either activating or degrading in nature.\textsuperscript{41,43} Organic nitrates are prodrugs that release NO, S-nitrosothiol or some NO-based moiety after biotransformation. Classically, these drugs have been considered to be endothelium-independent i.e. they do not rely on functioning endothelium for their vasodilator activity. In 1976, Needleman reported that GTN biotransformation requires reduced sulfhydryl groups\textsuperscript{44} but the mechanism of interaction between GTN and reduced sulfhydryl-containing cellular receptors remained a mystery for many years to come. More recently, Chen et al. provided evidence that the mitochondrial isoform of aldehyde dehydrogenase (ALDH2) is an important component of GTN bioactivation.\textsuperscript{45} This observation has been supported by further studies where GTN-dependent relaxation and blood pressure lowering is impaired in ALDH2 deficient mice. These experimental results have been confirmed by human studies showing an impaired GTN bioactivation and vasodilator potency on the setting of the genetic variant where ALDH2 activity is deficient (East Asian Glu504Lys point mutation) or where ALDH2 is pharmacologically inhibited (disulfiram).\textsuperscript{43} However, animal studies have demonstrated that inhibition of ALDH2 by benomyl did not completely eliminate vasodilation in response to GTN, but rather shifted the concentration-response curve to the right.\textsuperscript{46} This finding demonstrates that ALDH2 does not account for all GTN bioactivation and that two independent pathways appear to be involved. It has been proposed that ALDH2 is responsible for the biotransformation of low, therapeutic concentrations (in the nM range) of GTN while other enzymatic pathways are responsible for the biotransformation of GTN at higher (μM) concentrations. A number of enzymes have been proposed for this low affinity/high concentration pathway including, but not limited to, xanthine oxidase, and cytochrome P450 (CYP450). In addition, CYP450 is believed to be the primary pathway involved in the bioactivation of other organic nitrates including isosorbide dinitrate (ISDN) and isosorbide mononitrate (ISMN).\textsuperscript{43}
Figure 1.5. Proposed pathways of organic nitrate bioactivation in the vasculature. Bioactivation of high potency nitrates such as GTN, when given at low clinically relevant doses, is via mitochondrial ALDH2. This converts the organic nitrates to nitrite and denitrated metabolite. Nitrite in turn is bioactivated and yields NO which is able to activate the target enzyme, sGC. The increase in cGMP which results from activation of sGC, leads to decrease in intracellular calcium. Bioactivation of low potency nitrates such as ISMN and ISDN is via CYP450 enzymes in ER which yields NO directly. This figure is modified from Clin Res Cardiol 2008;97:12-20.

1.4.2. Nitrate tolerance:
Although nitrates are effective for treatment of cardiovascular disorders, development of tolerance i.e. the loss of vasodilatory and/or anti-ischemic effect after sustained therapy limits the therapeutic value of these drugs. The phenomenon of nitrate tolerance was first described in a case report in 1888, soon after a publication by Murrell in 1879 who reported that the oral administration of GTN relieved angina and prevented subsequent attacks. Although the phenomenon of tolerance has been recognized for more than a century, its systematic evaluation in clinical settings only began to be investigated 35-40 years ago. It is now well known that when nitrates are administered using dosing regimens or preparations that lead to continuous therapeutic plasma concentrations, their anti-ischemic and hemodynamic effects are lost within 24 hours. This has been documented in multiple studies, with various nitrate preparations even when very large doses are utilized. In the setting of congestive heart failure, continuous exposure to nitrates leads to attenuation of its haemodynamic effects. Of note, nitrate tolerance is independent of the route of administration and/or dose of ant given nitrate as it cannot be overcome with dose increases.
1.4.3. The mechanisms of nitrate tolerance and nitrate-induced endothelial dysfunction:
Although the mechanism underlying the nitrate tolerance is unclear, altered pharmacokinetics can be excluded as the cause since continued nitrate therapy leads to plasma concentrations that are similar to or higher than those during initial therapy. Many hypotheses have been proposed to account for nitrate tolerance. The most important hypotheses include: (1) sulphydryl depletion; continued therapy with nitrates leads to depletion of reduced sulphydryl groups which are necessary for biotransformation of nitrate to nitric oxide, (2) neurohormonal activation; administration of nitrates is associated with reflex release of vasoconstrictor hormones that counteract the vasodilating effect of nitrate, (3) plasma-volume expansion; continued nitrate therapy results in expansion of plasma volume which reverses the effects of nitrates on ventricular preload, and (4) free-radical production; nitrate administration leads to increase in production of free-radical by endothelium, which inhibits the activity of NO released by the nitrate. The mechanism by which nitrates lead to free-radical production is unclear but evidence suggests a role for angiotensin II (Ang II). The oxidative stress concept of nitrate tolerance was first reported in 1995 by Münzel et al. Later, mitochondria were identified as the source of oxidative stress in tolerant animals. It’s believed that mitochondrial respiratory chain is the primary source of nitrate-induced overproduction of O$_2^*$ in vessels, which leads to activation of vascular NADPH oxidase. It has been suggested that the activation of NADPH oxidase occurs secondary to stimulation of rennin-angiotensin-aldosterone system by GTN administration and subsequent increase in Ang II and aldosterone. Activation of NADPH oxidase in turn leads to further O$_2^*$ production. High concentrations of O$_2^*$ can overcome scavenging antioxidant enzymes like SOD and rapidly react with NO to form peroxynitrite. The increase in ROS production induced by nitrates are believed to play a causal role in the development of nitrate tolerance by at least three mechanisms: (1) peroxynitrite inhibits the activity of eNOS, reducing the bioavailability of endogenous NO, (2) peroxynitrite inhibits sGC and PKG leading to desensitization of signalling pathway of nitrates, and (3) ROS inactivate ALDH2 resulting in reduced bioactivation of the nitrate. The oxidative stress hypothesis of nitrate tolerance is supported by studies documenting that tolerance is prevented or reduced by co-administration of antioxidants such as vitamin C, folic acid and by inhibition of NADPH oxidase-dependent formation of ROS (statins, ACEIs, hydralazine). Interestingly, these antioxidant interventions are known to protect against endothelial dysfunction caused by oxidative stress. A number of studies reported that GTN therapy is associated with development of endothelial dysfunction in both coronary and peripheral human circulations. This effect was demonstrated to be prevented by antioxidant therapy. This provides evidence that O$_2^*$-mediated inactivation of NO is a mechanism common to both nitrate tolerance and clinical endothelial dysfunction.
Although there is a great deal of evidence supporting the oxidative stress hypothesis of nitrate tolerance, not all studies confirm this hypothesis.\textsuperscript{59}

### 1.5. Assessment of endothelial function

Despite extensive studies and development of several risk prediction models, traditional/conventional risk factors fail to predict a substantial proportion of cardiovascular events.\textsuperscript{60} As mentioned above, endothelial dysfunction is felt to play an important role in the development of atherosclerosis. Further, a number of studies have demonstrated that measured abnormalities in endothelial function serve as independent predictors of cardiovascular events in patients with both coronary artery\textsuperscript{61,62} and peripheral arterial disease\textsuperscript{63,64}. In addition, measures of endothelial function have been used to assess the impact of a number of pharmacologic interventions on vascular function in patients with pre-existing cardiovascular disease.

The assessment of endothelial function in humans has evolved over time with initial studies focussing on the coronary circulation.\textsuperscript{65} The evaluation of endothelial function relies upon the use of endothelium-dependent vasoactive compounds (such as acetylcholine) or interventions that stimulate the endothelium by increasing local shear stress. Previous studies have demonstrated an association between impaired endothelium-dependent dilation in coronary circulation with coronary atherosclerosis\textsuperscript{66} as well as coronary risk factors\textsuperscript{67}. Impairment in coronary endothelial responses has been documented in patients with CAD\textsuperscript{66,68,69}, heat failure (HF)\textsuperscript{70}, hypercholesterolemia\textsuperscript{71}, and hypertension\textsuperscript{71}. In humans, the coronary vasodilator response to ACh can be measured in the catheterization lab and is considered as the method with the greatest clinical relevance. Some studies have demonstrated that assessment of coronary endothelial function measures may have prognostic importance.\textsuperscript{72} Despite the advantages of coronary endothelial assessment, it is not suited to large population studies because it’s invasive, time consuming, and it cannot easily be repeated. However, a number of studies have proposed a link between the presence of endothelial dysfunction in coronary and peripheral circulations.\textsuperscript{73-76} Importantly, a study by Anderson et al. has demonstrated that patients with impaired coronary endothelial response had abnormal brachial arterial responses to reactive hyperemia and that there was a strong correlation between the responses in the two vascular beds. This finding has led to the concept that peripheral measures of endothelial function can be used as a surrogate for abnormalities in the coronary circulation. This, in turn, has led to the widespread use of non-invasive techniques such as flow-mediated dilatation (FMD) to assess endothelial function in
peripheral conduit arteries. Of note, some studies have now demonstrated that impaired FMD in the brachial artery has shown to independently predict short-term cardiovascular events.

1.5.1. Flow-mediated dilation:
Among techniques used for assessment of endothelial function, flow-mediated dilation (FMD) is the most widely used. The FMD technique was developed in 1992 as a non-invasive method for surrogate measurement of vascular endothelial function. FMD has gained favour due to its non-invasive nature, reproducibility, and the fact that it has been shown to correlate with coronary endothelial function. It has been used extensively to study mechanism of endothelial function, its response to interventions as well as to study differences in endothelial function in populations that are different based on age, sex, and CAD risk factors.

1.5.1.1. FMD measurement: The foundation of the technique is based on the capacity of blood vessels to respond to increases in blood flow, or more precisely a sudden increase in shear stress, by causing relaxation of the smooth muscle cells within the vascular media, which leads to vasodilation. It uses ultrasound-based imaging of forearm conduit vessels and their response to increases in shear stress as a measure of endothelium-dependent responses. FMD is performed by imaging the brachial or radial artery at rest using high resolution ultrasound imaging. Blood flow is interrupted for 4-5 minutes by inflation of a cuff distal to the site imaged. Release of the cuff causes hyperemia that is accompanied by an increase in shear stress parallel to the long axis of the blood vessel. The shear stress stimulus is transduced to the endothelial cell via mechanoceptors located in the endothelial surface causing the hyperpolarization of the cellular membrane, which in turn leads to opening of Ca²⁺ channels. This event leads to subsequent G protein activation of phosphokinase A, signaling an increase in eNOS activity (Figure 1.6). FMD is believed to represent a functional bioassay for NO bioavailability but also reflects production of other endothelium-derived vasodilators like prostaglandins and EDHF. This technique can be safely applied to healthy individuals and patients and can be repeated over time.
Endothelial nitric oxide synthase (eNOS) catalyzes the oxidation of L-arginine to produce nitric oxide (NO). NO diffuses into the vascular smooth muscle and activates soluble guanylate cyclase (sGC), which stimulates the conversion of GTP into cGMP. Cyclic GMP in turn mediates a decrease in intracellular calcium concentration causing vasorelaxation. Increase in flow leads to an increase in shear stress which leads to formation of NO. Intra-arterial infusion of acetylcholine (ACh) also leads to increased production of NO and a dose-dependent vasodilation.

This figure is modified from Annals of Medicine.2008;40:180-196.

1.5.2. Prognostic significance of FMD:

It is now well-established that endothelium-dependent vasodilator responses in both the forearm conduit and epicardial coronary artery distributions are impaired in patients with cardiovascular disease and cardiac risk factors.77,78 Although a large number of studies have documented a relation between FMD and cardiovascular risk factors, such a relationship has not always been demonstrated.84,85 An inexpensive and non-invasive method such as FMD would be advantageous in cardiovascular disease risk assessment. However, despite the strengths of FMD, it is still unclear whether it provides clear additional information to the prognostic accuracy of traditional cardiovascular risk factors. Some studies examining this question suggest that FMD provides only a marginal increase in prognostic information as compared to that provided by the traditional Framingham Risk Score in the prediction of cardiovascular events.20 Impaired brachial FMD has been shown to be a predictor of cardiovascular events in patients with CAD86, HF87, and postmenopausal women88. A study by Gokce et al. reported that impaired brachial FMD is an independent predictor of short-term cardiovascular events in patients undergoing vascular surgery.76 In addition, FMD measurement may also be of predictive value in older89 and middle-aged adults90 without clinical cardiovascular disease. Several other studies addressed the question of whether assessment of FMD
would provide prognostic information about cardiac events have yielded inconsistent results.\textsuperscript{78,91-93} Although many studies demonstrated a relation between FMD and cardiac events, not all studies demonstrated FMD to be predictive of cardiovascular events. Therefore, the ability of FMD to independently predict prognosis has not been fully resolved.

\subsection*{1.5.3. Limitations of FMD:}
Since FMD is expressed as a percentage change over a baseline, it is influenced by baseline arterial diameter. The baseline arterial diameter has been assumed to be consistent among all the individuals undergoing the measurement.\textsuperscript{94} However, it is possible that in certain circumstances FMD is blunted because of baseline conduit vessel dilatation.

Furthermore, the measurement of FMD only provides information concerning the recruitability of endothelium-dependent responses. That is, it is a measure of the ability of the endothelium to respond to a specific stimulus. However, it does not provide information about the resting endothelial activity (the activity of the endothelium prior to introduction of the FMD stimulus).\textsuperscript{20} From this perspective, if an artery has been stimulated (for example by exercise, emotional stimuli or the use of a drug), it is possible that FMD may appear blunted, despite the fact that endothelial function is intact. As such, in certain circumstances the presence of a blunted FMD cannot be taken as definitive evidence of abnormal endothelial function.\textsuperscript{94} This limitation of FMD has been overcome by introduction of a new index for assessment of response to low flow named low-flow-mediated constriction (L-FMC).\textsuperscript{20}

\subsection*{1.5.4. Low-flow-mediated constriction:}
Interruption of blood flow by cuff inflation during FMD measurement causes a reduction in shear stress which is associated with a reproducible vasoconstriction, generally 5-6\% of resting arterial diameter in healthy volunteers. This vasoconstriction is proportional to reduction in blood flow associated with cuff inflation.\textsuperscript{20} Of note, this constriction occurs, despite the fact that arterial distending pressure remains unchanged as the occlusion cuff is downstream to the site of imaging (at the level of the wrist). This phenomenon is now termed low-flow mediated constriction and has been the subject of several recent investigations concerning both its mechanism and its relationship to other measures of vascular function. Interestingly, the mechanism of L-FMC appears to be more complex than that of FMD. In a series of human studies, the mechanism of L-FMC has been investigated in an effort to determine the mediators involved. Administration of fluconazole (an inhibitor of the isoform 2C9 of the cytochrome P450, which is believed to be involved in the synthesis
of EDHF) and acetylsalicylic acid ((ASA), an inhibitor of PG synthesis) both caused significant blunting of L-FMC in healthy young volunteers while FMD was unaffected. Another investigation, by Spieker et al., demonstrated that inhibition of ET_{A} receptor by infusion of BQ-123 significantly reduced the vasoconstriction during cuff inflation while FMD was unaltered. This reduction in vasoconstriction identifies that ET_{A} receptor activation by ET-1 is an important underlying mechanism. Together, these data suggest that EDHF, PG and ET-1 are mediators involved in the regulation of resting endothelium-dependent vascular tone. Figure 1.7 demonstrates the mechanism involved in L-FMC. Of importance, the secretion of these mediators is abnormal in the setting of smoking, hypertension, diabetes and coronary artery disease.

Figure 1.7. A decrease in blood flow during cuff inflation occurs which reduces shear stress. L-FMC is determined by production of ET-1, EDHF, and PGs with ET-1 paying the major role. This figure is modified from European Heart Journal 2011;32:784-7.
It has been hypothesized that L-FMC provides information that is both additive and complementary to that provided by FMD. Although a large number of studies have used FMD as a tool to test physiologic, pharmacologic and clinical endpoints, it is clear that there are limitations to this technique as a measure of endothelial and vascular health. While technical limitations (use of a probe holder, distal ischemia versus proximal ischemia, use of very high resolution systems and computerized analysis systems) have largely been overcome, a systematic limitation remains to be addressed. The recognition that FMD is a measure of the ability of the endothelium to respond to a specific stimulus but not a measure of resting endothelial activity has emphasized the potential for the FMD technique to underestimate endothelial responses in certain circumstances. When FMD is measured in the setting of a stimulated/activated endothelium it is possible to conclude, incorrectly, that the function of the endothelium is abnormal since any further response to increases in shear stress will be depressed. A good example is what occurs in the case of normal volunteers who undergo exercise with repeated handgrip. Following exercise there was an increase in baseline blood flow and arterial diameter with a blunting of FMD but an increase in L-FMC. Of note when on summates the absolute values for both FMD and FMC their value before and after exercise is unchanged, despite changes in the value of constituent scores. Therefore, the information provided by measurement of L-FMC allows for quantification of basal activity of endothelium providing information that is complementary to traditional FMD measures. Combining information obtained from traditional FMD, which is information that express endothelial reserve, with those obtained from L-FMC, the resting endothelial activity, should improve sensitivity and specificity of endothelial function assessment, enhancing the utility of this technique in both mechanistic and clinically relevant studies. A previous study reported that the repeatability and reproducibility of L-FMC are similar to those reported for FMD. As with FMD, assessment of L-FMC is non-invasive, simple, reproducible, and does not need extra procedures as compared to traditional FMD. Importantly, L-FMC is independent of changes in blood pressure and/or of autonomic stimuli.

The availability of data on the resting endothelial activity led to introduction of a third parameter called composite end point. This parameter is calculated as the sum of the absolute value of L-FMC and the absolute value of FMD for each individual. The composite end point reflects the overall activity of arterial wall and is a better predictor of endothelial dysfunction and vascular diseases. Discriminating patients with hypertension, congestive heart failure (CHF), and coronary artery disease (CAD) from healthy individuals is significantly improved using this parameter.
Representative plot of radial artery diameter measurement using FMD technique. A decrease in blood flow subsequent to cuff inflation occurs which causes a progressive decrease in arterial diameter (FMC). Upon cuff deflation, the enhanced blood flow leads to increased shear stress which in turn causes arterial dilatation. FMC is calculated as percentage decrease in arterial diameter compared to baseline diameter. FMD is determined as percentage increase in arterial diameter following cuff deflation as compared to baseline diameter. Composite end point is calculated as the sum of FMC and FMD.

1.5.5. Plethysmography:
Venous occlusion plethysmography, first described in 1909, is one of the oldest methods to assess peripheral blood flow in humans. The technique has continued to be widely applied in human studies and has remained essentially unchanged except for the advent of computerized data acquisition and analysis techniques. The underlying principle of venous occlusion plethysmography is straightforward; when venous outflow from the arm is briefly obstructed and arterial inflow continues unimpeded, blood can enter the forearm but cannot escape. This leads to a linear increase in forearm volume at a rate proportional to the rate of arterial inflow. Under resting conditions, 50-70% of the total forearm blood flow is through skeletal muscle and most of the remainder through the skin. The circulation of the hand is typically excluded from the measurement since the hand circulation contains...
a large arteriovenous shunt, is very sensitive to temperature variations, and has a different physiology and pharmacology than muscle blood flow. In practice, exclusion of hands from circulation is achieved by placing a high pressure pneumatic cuff at the wrist and inflating it to suprasystolic pressure. A cuff placed on the upper arm is inflated to a pressure above venous pressure but below arterial diastolic pressure, generally 40mmHg. With the upper arm cuff inflation, forearm volume increases linearly and is proportional to arterial flow. This relationship between increase in forearm volume and arterial inflow holds true only if the veins are not fully distended. Once the veins are fully distended, continued arterial inflow causes increasing venous pressure and eventual loss of fluid from the vascular space because of increased hydrostatic pressure with consequent loss of the proportional relationship between forearm volume and arterial inflow. To prevent this, upper cuff inflation is limited to 10 seconds followed by a 10 second cuff deflation to permit venous drainage. The forearm must be positioned above the level of the heart to allow emptying of the forearm veins during the period of deflation.97,98

The method is based on the principle that changes in forearm volume can be estimated from changes in forearm circumference, assuming that the increase in forearm circumference occurs evenly along the length of the forearm and that the forearm length remains the same. The changes in the forearm volume that occur upon upper arm cuff inflation are measured by mercury-in-silastic strain gauge plethysmography. The strain gauges must be placed around the widest part of the forearm and act as resistors. The increase in forearm volume leads to corresponding increase in arm circumference and thus the length of the strain gauge. This is detected by the strain gauge as an alteration in electrical resistance and therefore a change in electrical potential. The blood flow is expressed per unit volume of forearm; milliliters per 100mL forearm per minute.97,98

This plethysmographic technique is often combined brachial artery drug infusions. This allows study of vascular physiology and pharmacology by infusion of receptor antagonists and agonists, respectively. Commonly used vasoactive agents in plethysmographic studies are ACh, bradykinin, substance P, and NOS inhibitors.

1.6. Treatment of endothelial dysfunction

The hypothesis that the development of endothelial dysfunction occurs early and is important in the development of overt vascular disease led to the concept that therapy which was associated with normalization of endothelial function might be associated with long-term clinical benefit. Although studies directly testing this hypothesis have yet to be performed, mounting evidence suggests that
treatment strategies, if implemented early, could limit cardiovascular events. Interventions such as exercise, dietary modifications, antioxidant therapy, lipid-lowering therapy, ACEIs, and angiotensin receptor blockers (ARBs) have been associated with improved endothelial function.\textsuperscript{99-102} Of note some agents, particularly HMG CoA reductase inhibitors and ACEIs, have been shown to improve endothelial function but are also associated with substantial improvements in clinical outcome.\textsuperscript{103,104} Although a casual link has never been established, this combination of observations suggests that improvements in endothelial function could serve as a surrogate endpoint predicting improvements in clinical status.

1.6.1. Prevention of IR-induced endothelial dysfunction:

1.6.1.1. Ischemic preconditioning: In 1986, Murry and co-workers observed that brief, intermittent episodes of ischemia prior to a sustained bout of ischemia have a protective effect on myocardium. In this study, authors exposed the test animals to four, 5 minute episodes of ischemia each followed by a 5 minute episode of reperfusion (ischemic preconditioning (IPC)) prior to a 40 minute period of coronary occlusion. In the preconditioned animals myocardial infarct size was only one-fourth the size of infarcts found in control animals (those without preconditioning). This effect was found to be independent of coronary collateral blood flow.\textsuperscript{105} The phenomenon of IPC has been observed in a number of organ systems in both animals and humans.\textsuperscript{106} IPC consists of two chronologically and pathophysiologically distinct phases; an early window, which develops very quickly and lasts for 1-4 hrs and a late window, which develops slowly (6-12 hrs) and lasts for up to 72 hrs. The mechanisms of the protective effects observed in the two preconditioning windows are different. The early window is caused by post-translational modification of pre-existing proteins while the late window is caused by production of new cardioprotective proteins. The mechanisms involved in IPC are complex and have been the subject of intense investigation.\textsuperscript{107} Adenosine has been suggested to play role in the protection afforded by early window of IPC. However, other endogenously released mediators such as catecholamines, bradykinin, opioids, and ROS are generated during preconditioning all of which have appear to play a role.\textsuperscript{108} The stress of the preconditioning stimulus causes release of chemical signals that act as triggers of the late window of preconditioning process. A number of triggers have been identified which include NO (generated by eNOS), ROS, endogenous opioids, and adenosine. These chemical signals lead to activation of a complex signal transduction cascade that includes PKC-\varepsilon, the Src/Lck isoforms of tyrosine kinases, Janus-activated kinases 1 and 2 (JAK1/2). Activation of these signalling pathways results in activation of cytoplasmic and normally dormant stress-responsive transcription factors such as NF-\kappaB, STAT1, and STAT3. The exact mechanisms involved remain incompletely understood and involvement of other as yet unidentified protein kinases and
transcription factors is likely. In general this process results in upregulation of cardioprotective genes, leading to formation of new proteins that mediate the protection afforded by late PC. The first gene identified as a mediator of late PC was iNOS; subsequently, other genes have been discovered to be essential in mediating this response including COX-2, heme oxygenase (HO)-1, and antioxidant enzymes such as extracellular SOD, aldose reductase, and manganese SOD. Heat stress proteins have also been suggested to contribute to late PC but this remains to be uncertain.\(^\text{107}\) It is worth noting that while all of the factors mentioned are important in mediating the late IPC response, their exact relationships remain poorly understood. In addition, these lines of evidence have been developed in animals and further studies are required for detection of their exact role in preconditioning in humans.

1.6.1.2. Pharmacologic preconditioning: Certain pharmacologic agents have been shown to induce a phenotype that is very similar to that observed with IPC. This phenomenon, known as pharmacologic preconditioning, appears to occur through mechanisms similar to those seen with IPC and has been observed in myocardium, endothelium, and a number of other tissues. A broad range of pharmacologic interventions have been shown to exert preconditioning effects in both the early and late window. Although their mechanisms of action remain incompletely understood, in most cases pharmacologic preconditioning agents appear to trigger the same protective pathways involved in classic, ischemic preconditioning. For example, sildenafil and GTN have been shown to have pharmacologic preconditioning effects by acting on the NO/cGMP axis. In a study from our laboratory, sildenafil was shown to limit blunting of FMD after an IR episode in healthy humans. This effect of sildenafil was demonstrated to be through opening of \(K_{\text{ATP}}\) channels as blockade of these channels by glibenclamide prevented the endothelial protection by sildenafil.\(^\text{109}\) In another investigation Gori et al., demonstrated that short-term administration of GTN was associated with a late window preconditioning effect.\(^\text{110}\) Importantly this protective effect could be completely abolished by the administration of vitamin C during the period of GTN exposure, suggesting that the preconditioning effect was triggered by an increase in ROS production associated with GTN therapy.\(^\text{110}\) However, although the understanding of the pathophysiology of IR injury has improved in recent years and a wide range of preconditioning approached are under investigation, the use of preconditioning approaches has not yet been widely used in clinical practice.

1.6.2. Prevention of nitrate tolerance:
Several strategies have been tested to prevent development of nitrate tolerance. A number of pharmacologic interventions have been shown to be successful in both animal and human models,
however none of these approaches has been adopted into clinical practice. In order to avoid tolerance, the most widely approach used clinically is the intermittent nitrate therapy where either the dosing regimen or the nitrate formulation provides for a low or nitrate free interval for several hours each day. However, this intermittent therapy approach, with a nitrate-free interval, is problematic as patients who have been relatively free of ischemia during the nitrate therapy, may develop more ischemia during the nitrate free interval i.e. the patients are prone to rebound ischemia.\textsuperscript{111} This rebound ischemia maybe due to the development biochemical changes that make the coronary vasculature more sensitive to vasoconstrictors during nitrate therapy.\textsuperscript{112} During nitrate therapy, these biochemical changes are balanced by the dilating effect of the nitrate but during the nitrate free interval, the biochemical changes persist while it’s not opposed by the vasodilating properties of the nitrate.\textsuperscript{112}

A number of treatment strategies that specifically target presumed biochemical mechanisms of nitrate tolerance have been attempted. Based on the Needleman hypothesis that nitrate biotransformation required a supply of reduced sulfydryl groups and that tolerance resulted from their exhaustion, studies have been performed with supplemental thiol donors in an attempt to prevent tolerance. Studies in both animals and humans initially suggested that sulfhydryl donors such as N-acetylcysteine\textsuperscript{113,114} and L-methionine\textsuperscript{115} could prevent the development of nitrate tolerance. However, further studies demonstrated that the observed results actually represented an augmentation of nitrate effects rather than the true prevention of tolerance.\textsuperscript{116} Furthermore, carefully performed human studies demonstrated that supplemental N-acetylcysteine did not prevent tolerance to the anti-anginal effect of isosorbide dinitrate.\textsuperscript{117}

Based on the neurohormonal activation hypothesis of nitrate tolerance, a number of studies have investigated the effects of ACEIs and ARBs on nitrate tolerance. However, data concerning the effect of ACEI co-administration in preventing nitrate tolerance in CAD and HF have been mixed with positive\textsuperscript{118,119} and negative results\textsuperscript{120,121}. Evidence supporting the benefit of ARBs in the setting of nitrate tolerance have been inconsistent and conflicting.\textsuperscript{122,123} Although it is difficult to explain the conflicting results, the use of differences in the endpoints used in the assessment of nitrate tolerance and in the doses of the drugs used may have played a role.

More recent studies to prevent nitrate tolerance have focused on the free-radical production hypothesis. These studies have been designed to modulate the synthesis of ROS and/or NO. A number of interventions such as hydralazine\textsuperscript{124}, carvedilol\textsuperscript{125}, vitamin E\textsuperscript{126}, and vitamin C\textsuperscript{127,128} have been tested in animal and human models. These interventions have demonstrated the ability to prevent/reverse
nitrate tolerance. In a study by Gori et al. it was documented that supplemental folic acid prevents the development of both NOS dysfunction and GTN tolerance during continuous nitrate therapy in healthy volunteers. It has been hypothesized that the effect of folic acid on NOS dysfunction is mediated by tetrahydrobiopterin and is independent of synthesis of superoxide anion by xanthine oxidase and the direct antioxidant property of folic acid. Despite these successes, antioxidant therapy has never been adopted into common clinical practice. However it is interesting that the combination of isosorbide dinitrate has been shown to improve clinical outcome in patients with chronic heart failure although whether this is due to an antioxidant effect of hydralazine has never been clear.

1.7. Beta-blockers
Beta-blockers play an important role in the management of many cardiovascular conditions including such as hypertension, coronary artery disease, and chronic heart failure. Their main mechanism of action is through blockade of cardiac β-adrenergic receptors and leading to decreased force and rate of heart contraction with subsequent reduction in arterial blood pressure and cardiac load. In the early 1960s, James Black and colleagues hypothesized that beta-blockers such as pronethanol and propranolol would interfere with catecholamines and lower myocardial oxygen consumption, and therefore would be useful for the treatment of angina pectoris, hypertension, and arrhythmias. Even though pronethanol was proved to be effective in treatment of angina pectoris, propranolol became the prototype beta-blocker and was rapidly adopted into the clinical management of angina and hypertension. In fact propranolol was the first major advance in the therapy of angina since the introduction of GTN almost 100 years earlier. Beyond angina it was soon recognized that propranolol (and other beta-blockers which followed) were effective in the treatment of patients with arrhythmias, hypertension, and hypertrophic cardiomyopathy.

The potential adverse reactions associated with propranolol on heart rate, myocardial contractility, and bronchial tone led to modifications in pharmacologic structure of beta-blockers. The advancement in drug development led to introduction of new β-blockers with greater selectivity for β₁-adrenergic receptors and a lower incidence of side effects (so-called second generation β-blockers). Blockade of β-receptors in peripheral vasculature can lead to vasoconstriction and an increase in peripheral resistance. Although this is generally not a clinically significant effect it can be problematic in the setting of sever peripheral vascular disease. Therefore, β-blockers with additional properties have been developed in an effort to prevent this effect and provide a peripheral vasodilator effect. Several new compounds have been developed (third generation β-blockers) with different pharmacologic properties. An important and widely used third generation β-blocker is carvedilol.
which, in addition to its β-blocking effects, also has \( \alpha_1 \)-adrenergic receptor blocking effect. Carvedilol has been shown to reduce mortality in patients with heart failure and appears to have a greater impact on mortality than traditional β-blockers.

Another third-generation β-blocker, nebivolol, is a \( \beta_1 \)-cardioselective β-blocker with additional vasodilator properties. It has been used effectively in patients with hypertension, heart failure, and left ventricular dysfunction. Nebivolol is a lipophilic β-blocker consisting of a 1:1 racemic mixture of \( d \)- and \( l \)-nebivolol. The recommended starting dose is 5mg daily for most patients and if further reduction in blood pressure is required, the dose can be increased to 20mg daily. Nebivolol’s pharmacokinetic steady state is reached in 3 and 5 days in CYP2D6 extensive and poor metabolizers, respectively. Approximately, 98% of nebivolol is bound to plasma proteins, mostly albumin, and it is widely distributed into tissues including brain. Nebivolol is predominantly metabolized by CYP450 2D6. Its stereospecific metabolites contribute to its pharmacologic activity. The half-life of nebivolol is 13 hours in extensive metabolizers of CYP 2D6 and 22 hours in poor metabolizers. After a single oral dose, 37% of the dose was recovered in urine and 42% in feces for extensive metabolizers and 57% in urine and 8% in feces for poor metabolizers.

Nebivolol reduces peripheral vascular resistance, an effect that appears to be mediated by several mechanisms. A number of mechanisms have been proposed for nebivolol-induced relaxations including estrogen receptor-dependent eNOS translocation, phosphorylation of the serine 1177, and stimulation of serotonin receptors. An intriguing hypothesis is that nebivolol is a \( \beta_3 \)-receptor agonist and that this activity stimulates the activity of eNOS. It is believed that most important mechanisms of nebivolol-induced vasodilation are; (1) activation of eNOS via binding of nebivolol metabolite to \( \beta_2 \)-receptor; Broeders and colleagues demonstrated that nebivolol per se is unable to increase the production of NO; however, when nebivolol was allowed to metabolize, the addition to aortic tissue induces the release of NO and augments the intracellular calcium concentration in endothelial cells, processes that are \( \beta_2 \)-adrenergic receptor-mediated and endothelium-dependent, (2) direct binding of nebivolol to \( \beta_3 \)-receptor; human and rodent coronary microvessel studies has documented that nebivolol-induced vasodilation is via endothelium-dependent hyperpolarization and NO. This effect was abolished by eNOS inhibition, blocked by \( \beta_3 \)-inhibitors, and absent in \( \beta_3 \)-knockout mice, and (3) stimulation of endothelial ATP-efflux; nebivolol activates endothelial ATP-efflux, increasing endothelial calcium level via P2Y-receptors and leading to calcium-dependent activation of eNOS.
A double-blind, parallel group study by Lacourciere et al. observed a significant decrease in total cholesterol and LDL after nebivolol treatment rather than the expected increase as with traditional β-blockers.\(^{129}\) Similarly, a number of studies have shown that nebivolol improves insulin sensitivity, oxidative stress, plasma adiponectin, and soluble selectin plasma levels in patients with hypertension and/or diabetes mellitus.\(^{140-143}\) All of these beneficial metabolic effects of nebivolol appear to be mediated through stimulation of β\(^3\)-receptors.\(^{132}\)

In a study by Mollmau et al.\(^{144}\) eight weeks treatment of nebivolol led to a significant inhibition of superoxide in blood vessels. Reductions in oxidative stress within the vasculature caused a significant improvement in endothelium-dependent and -independent responses to ACh and GTN, respectively. This study documented an inhibitory effect of nebivolol on angiotensin II-induced superoxide production and on PKC-dependent activation of NADPH oxidase. By suppressing NADPH oxidase activity and by inhibiting tissue infiltration by inflammatory cells, nebivolol directly increases vascular NO bioavailability and reduces potent oxidant peroxynitrite formation. Oxidant peroxynitrite has been hypothesized to play a role in eNOS uncoupling. Therefore, nebivolol has shown to have antioxidant properties and beneficial influence on the progression of atherosclerotic process.\(^{144}\)

These effects of nebivolol are believed to be independent of its beta-blocking activity and involve increase in the production and/or bioactivity of NO. This is supported by a human study published in 2005, who observed that nebivolol but not atenolol, another selective β\(_1\)-adrenergic receptor blocker, improved endothelial dysfunction in hypertensive patients.\(^{165}\)

**Figure 1.9.** Proposed mechanisms of action of nebivolol.
1.7.1. Nebivolol and protection from IR injury:
A recent study by Heeba et al.\textsuperscript{145} investigated whether nebivolol has neuroprotective effect against cerebral IR injury in rats and also studied the mechanism of its neuroprotection. In this study, authors observed that nebivolol plays a neuroprotective role in acute cerebral IR injury as evident from significant decrease in cerebral infarct volume and amelioration of biochemical and histopathological status. In addition, nebivolol was shown to increase eNOS expression with a simultaneous decrease in iNOS expression in a dose dependent manner after IR insult in rats’ brain tissue. Results of this study revealed that nebivolol is able to protect against GSH (reduced glutathione) depletion, increase lipid peroxides, and elevate SOD activities induced by IR insult, therefore confirming its ability to suppress ROS formation secondary to IR injury.\textsuperscript{145} In another study, l-nebivolol was shown to have beneficial effects on endothelial dysfunction induced by IR. The authors hypothesize that the effectiveness of l-nebivolol in reducing ischemic injury is via NO production.\textsuperscript{146} These studies suggest that nebivolol may have the ability to be used as a pharmacologic preconditioning agent.

1.7.2. Nebivolol and protection against nitrate tolerance:
As mentioned previously, nitrate therapy leads to increased production of $O_2^\bullet$, activation of NADPH oxidase, further increase in $O_2^\bullet$ production. The latter reacts with NO and leads to generation of peroxynitrite. Peroxynitrite, in turn, suppresses the activity of eNOS and therefore leads to decreased bioavailability of NO.

However, nebivolol has been shown to suppress NADPH oxidase activity, reduce potent oxidant peroxynitrite formation, and increase NO bioavailability. Based on these observations, we hypothesized that nebivolol might have the ability to protect the blood vessels against tolerance induced by continuous nitrate therapy.
Assessment of endothelial function using flow-mediated constriction in the setting of acute systemic inflammation

2.1. Abstract:

Objective: The aim of the present study was to investigate the role of L-FMC, alone and in combination with FMD in response to systemic inflammation caused by vaccination. We hypothesized that the induction of systemic inflammation would blunt FMD but also increase FMC because vascular inflammation would increase resting vascular tone.

Background: Previous papers that have studied the effect of inflammation on FMD, reported a blunted FMD after vaccination and based on this finding, concluded that inflammation leads to endothelial dysfunction. None of these studies took FMC into consideration and the blunted FMD that they have reported might be an expression of inability of a stimulated endothelium to respond to further stimuli and not endothelial dysfunction.

Method: 30 volunteers were enrolled in a double-blind, parallel study. At baseline, FMD of the radial artery was measured, a venous blood sample was obtained, as well as sitting heart rate and blood pressure. Subjects were then randomized, in an investigator blind fashion, to receive an intramuscular injection of Salmonella typhi capsular polysaccharide vaccine 0.025mg or placebo (normal saline). Measurements were repeated 4, 8, and 24 hours after randomization.

Results: The study had to be stopped prematurely because the Salmonella typhi vaccine was withdrawn from the market. In the 30 subjects which completed the study (15 in each group) there was no significant change in either FMD or FMC in either group. In the group receiving the vaccine there was a statistically significant increase in CRP 24 hours after vaccination ($P=0.0005$). There was no change in the inflammatory biomarker IL-6 in either group.

Conclusion: We found that the administration of the Salmonella typhi vaccine did not alter endothelial function in the radial artery. We did find an increase in CRP in response to the vaccine. It is clear that our findings may have underestimated the impact of a systemic inflammatory response on vascular function since the vaccine employed was withdrawn as quality assurance testing by the manufacturer had revealed that it displayed inadequate antigenicity responses.
2.2. Introduction:
While performing FMD measurements, the increase in vascular resistances caused by the inflation of the wrist cuff leads to a progressive reduction in blood flow in the segment of the artery that is proximal to the cuff. This reduction, which reaches a factor of 50-75% of the initial flow, is associated with a parallel reduction in shear stress at this level. In turn, this leads to a vasoconstriction of 5-6% of the resting arterial diameter in healthy volunteers.

We hypothesized that the measurement of FMC would provide data that are additive to those of FMD. Although the number of studies which have used FMD as a tool to test physiologic, pharmacologic and clinical endpoint bears witness to its success, it is clear that there are limitations to this technique as a measure of endothelial and vascular health. While technical limitations (use of a probe holder, distal ischemia versus proximal ischemia, use of very high resolution systems and computerized analysis) have largely been overcome, a systematic limitation remains to be addressed: this is best summarized by recognizing that FMD is the expression of endothelial reserve (that is, it is a measure of endothelial recruitability) and not a measure of resting vascular endothelial tone. To state this in another way - the FMD method applied to a fully functional and stimulated endothelium (for instance a healthy volunteer undergoing physical stress) can incorrectly conclude that the function of the endothelium is abnormal since any further response to increases in shear stress (the FMD stimulus) will be depressed. To date, the assessment of such basal ‘resting’ endothelial activity can only be made invasively by infusing specific inhibitors. The critical additional information brought by the measurement of FMC is that this variable allows us to quantify the basal activity of the endothelium non-invasively. As such, FMC provides information that is both complementary to the results of FMD measurements. Combining information about endothelial reserve provided by FMD with information concerning resting endothelial activity provided by FMC should improve the utility of this non-invasive technique in the assessment of endothelial function.

The aim of the present study was to investigate the use of FMC, alone and in combination with FMD in the assessment of conduit arteries. In this study we set out to determine whether the blunted FMD in setting of inflammation is an indication of endothelial dysfunction or rather a reflection of increased vascular tone/release of dilating autocoids. Therefore, the questions to be addressed were; (1) Is FMC increased by stimuli that alter endothelial activity, such as acute inflammation; and (2) Can the analysis of FMD alone lead to inappropriate diagnosis of endothelial dysfunction?
2.3. Materials and methods:

The Mount Sinai Hospital Research Ethics Board approved this investigator initiated, non-industry funded study, and all subjects gave informed consent. Thirty healthy non-smoking volunteers (18 to 30 years old) were enrolled in a double-blind, randomized, placebo-controlled, parallel trial. Subjects were asked to abstain from caffeine, alcohol, and flavonoid-containing beverages and to fast for at least 12 hours before the study. Subjects were also required to refrain from any form of exercise for a week before participation. On study visit 1, sitting heart rate and blood pressure were obtained followed by venous blood sampling for analysis of inflammatory markers; interleukin-6 (IL-6) and C-reactive protein (CRP). Subsequently, radial artery FMD and FMC were measured. Subjects were then randomized to receive either *Salmonella typhi* capsular polysaccharide vaccine 0.025mg or placebo (normal saline) by intramuscular injection into the deltoid muscle. FMD measurement, blood pressure, and blood sampling was repeated 4, 8, and 24 hours after the injection. We employed an investigator-blind technique in which the randomization was performed by a research associate not involved in either data acquisition or analysis.

![Randomization diagram](image)

**Figure 2.1.** Diagram of the experimental design of the study. Blood pressure and FMD were measured at baseline and blood was withdrawn. Subjects were then randomized to either placebo injection or *Salmonella typhi* vaccine. The measurements were repeated 4, 8, and 24 hours after randomization.

2.3.1. *Salmonella typhi* capsular polysaccharide vaccine:

Two manipulations used frequently in experiments to induce inflammation are administration of low dose Escherichia coli endotoxin and *Salmonella typhi* vaccine. The advantage of *Salmonella typhi* vaccine is that it is approved by FDA and is associated with fewer side effects. This vaccine has been used in a number of studies as a model of inflammation. Increases in IL-6 have been documented 2 hours after vaccination with *Salmonella typhi* which sustained up to 12 hours. Increases in CRP level have been observed 24 hours post vaccination. However, vaccination is not associated with changes in blood pressure, heart rate, cardiac output, and total peripheral resistance. Therefore, vaccine-induced inflammation has no effects on cardiovascular parameters. *Salmonella typhi* vaccine is indicated for travellers to epidemic areas where the risk of exposure to contaminated food
and water is high. The side effects of the vaccine are categorized into local and systemic; local side effects include pain, edema, redness, and tenderness at the injection site, and the systemic side effects are headache, fever, and tiredness.

**Note:** After enrollment and randomization of 30 subjects, the *Salmonella typhi* vaccine was withdrawn from the market due to concerns that the vaccines would contain lower than expected antigen content. Therefore, this study had to be stopped prematurely.

### 2.3.2. Flow-mediated dilation and flow-mediated constriction:

A detailed description of the FMD technique has been published in review and guideline articles\(^{82,148}\). Briefly, the left radial artery is imaged while the subject was in supine position using a clinical ultrasound imaging platform (Vivid7, GE Healthcare) with a high-resolution linear array transducer (14MHz). During image acquisition, a probe holder and a deflation pillow were used to ensure stable transducer and arm position during the study. The end-diastolic, ECG-gated, longitudinal, B-mode images are digitally acquired using a personal computer and stored for off-line analysis. After ten minutes of rest, baseline resting arterial diameter and blood flow measurements are performed. Subsequently, a pneumatic cuff placed at the level of the wrist (i.e., distal to the site of arterial diameter measurement) is inflated to 250 mmHg for 4 minutes and 30 seconds. Upon release of the cuff, blood flow under the ultrasound transducer suddenly increases due to maximal vasodilation of the territories exposed to ischemia. This causes a parallel increase in shear stress at the level of the proximal radial artery, which caused the endothelium to release its vasodilator autacoids causing arterial dilatation. The radial arterial diameter is recorded continuously during baseline, cuff inflation, and for 4 minutes and 30 seconds after wrist cuff deflation. FMD is then calculated as the percent maximum increase of arterial diameter measured during the 4′ and 30″ period following wrist-cuff deflation as compared to resting arterial diameter. The whole procedure requires approximately 20 minutes (10 minutes baseline measurement, 4′30″ seconds cuff inflation, 4′30″ seconds diameter measurement after cuff deflation). Our laboratory has recently developed a completely automatic computerized system that allows continuous measurement of the arterial diameter through resting conditions, cuff inflation and after cuff deflation. This method measures FMD, a parameter of endothelial ‘recruitability’.

Of note, the measurement of FMC does not require any further procedures as this measurement is available from the imaging recordings made during standard FMD procedures. During cuff occlusion, in response to the reduction in blood flow and shear stress, arterial diameter decreases progressively
until it reaches a plateau. Our preliminary studies show that the plateau of this decrease in vessel size occurs approximately 3’ and 30” into cuff inflation. In order to standardize this measurement, we calculated FMC as the percent decrease in arterial diameter in the last 30” of cuff inflation as compared to resting diameter. Our hypothesis is that the concomitant measurement of FMC and FMD provides information concerning 1) how active is the endothelium in resting conditions (FMC) and 2) how much ‘recruitable’ endothelial function there is (FMD). Although we examine and report FMD and FMC responses separately we also intend to make use of the composite score – that is the total amount of FMD and FMC observed as this, we believe, provides the most complete measure of endothelial function (both recruitable and resting).

2.3.3. Statistical analysis:
Data are presented as mean ± SD. To calculate the number of subjects required to detect 40% change in the vaccine as compared to placebo group, we used a previously reported values for healthy volunteers after injection of Salmonella typhi vaccine. Vaccination decreased FMD from 6.9±1.3% to 3.5±0.6%. To get similar blunting of FMD after vaccination, a sample size of 20 per group is required. This sample size yields a 1-β=0.8 and a 2-sided α of 0.05. The data was analyzed using 2-way ANOVA for within-group and between-group comparisons. Post-hoc comparisons were performed with 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.

2.4. Results:
Thirty healthy individuals were randomized to receive Salmonella typhi vaccine or placebo (normal saline) by intramuscular injection. There were no significant differences in resting heart rate, blood pressure, resting radial artery diameter, baseline blood flow, reactive hyperemia, or baseline FMD between the 2 groups.

Table 1.1. Baseline characteristics of the study subjects. MAP: Mean Arterial Pressure.

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Vaccine</th>
</tr>
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<tbody>
<tr>
<td>Age (range)</td>
<td>22 (18-27)</td>
<td>23 (18-27)</td>
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<tr>
<td>SBP (mmHg)</td>
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<tr>
<td>FMD (%)</td>
<td>7.4±3.2</td>
<td>8.8±4.3</td>
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</table>
We did not observe a significant change in systolic blood pressure in either the vaccine or placebo group (Figure 2.2., \( P=\text{NS} \) for ANOVA effect of group, and for the interaction of time and group). There were no significant changes in diastolic blood pressure (Figure 2.3) and heart rate (Figure 2.4) in either group at any time point.

Figure 2.2. Systolic blood pressure in placebo and vaccine groups at four different timepoints. There is no evidence to show significance between the two groups.

Figure 2.3. Diastolic blood pressure at different time points in both groups. Vaccination did not change DBP significantly.
Vaccination with *Salmonella typhi* did not change FMD response in either group (Figure 2.5., *P*=NS for ANOVA effect of group, time, and for the interaction of time and group). Furthermore, vaccination with *Salmonella typhi* vaccine had no effect on FMC at any time point (Figure 2.6.).
Figure 2.6. FMC response in both the placebo and vaccine groups. Vaccination had no significant effect on FMC response.

There was a significant increase in the CRP 24 hours after the administration of the vaccine (Baseline CRP=944±680 ng/mL, 24 hours CRP=1900±1601 ng/mL, P=0.0005; Figure 2.7). CRP did not change significantly in the control group (Baseline CRP=924±1299 ng/mL, 24 hours CRP=1134±1399 ng/mL, P=NS; Figure 2.7). Importantly, there was no significant difference between groups in the CRP response. The plasma concentration of IL-6 did not change in either group (Figure 2.8).
Figure 2.7. The inflammatory marker, CRP, in the placebo and vaccine group at baseline and 24hrs after administration of placebo or vaccine. CRP did not increase significantly in the placebo group. Vaccination with *Salmonella typhi* vaccine caused a significant increase in CRP level at 24hrs. Data are mean ± SD. *P=0.0005 versus baseline vaccine.

Figure 2.8. The inflammatory marker, IL-6 in both the vaccine and placebo groups. IL-6 did not change significantly in either group.

2.5. Discussion:

The healthy vascular endothelium has vasodilator, anti-adhesive, anti-inflammatory and anti-coagulant properties. These properties of the endothelium are believed to be mediated by the
production of a variety of local autocoid mediators, including NO. Endothelial dysfunction is a state of reduced dilator function, increased inflammatory cell and platelet adhesion, and increased coagulation activity. Endothelial dysfunction is an early event in the pathogenesis of a number of cardiovascular diseases including atherosclerosis. Reduced bioavailability of NO makes a major contribution to endothelial dysfunction may occur because of reduced NO synthesis or increased NO degradation due to chemical reaction with oxidant radicals.

An important causal factor in various states of endothelial dysfunction is the presence of inflammation. Inflammatory cytokines have been shown to impair endothelial function in animal models and isolated human veins. A previous study demonstrated that acute systemic inflammation, induced by typhoid vaccination, leads to endothelial dysfunction. To date, three studies have documented that endothelial function, as measured by FMD, becomes abnormal following acute inflammation caused by vaccination. These reports conclude that acute inflammation is associated with the development of endothelial dysfunction. However, these prior reports did not examine the effect of acute inflammation on FMC. In the current study, we hypothesized that the blunted FMD caused by vaccination might represented a blunted response caused by activation of the conduit vessel endothelium in response to the inflammation and that these changes might be associated with an increase in FMC.

Present study was discontinued prematurely because the manufacturer of the vaccine recalled few batches of its typhoid vaccine due to inadequate antigenecity and unfortunately the vaccines used in this study were among the recalled batches. Although a number of subjects, who received the vaccine did have an elevation in CRP level indicating some degree of inflammation, the degree of inflammation was insufficient for the hypothesis to be tested. The power of 0.64 was achieved with the sample size of 15 indicating a high chance of type II error. Therefore, our findings might not be relevant.

2.5.1. Study limitations:
We found no significant effect of the Salmonella typhi vaccine on either FMD or FMC. Despite the fact that we did find evidence of systemic inflammation in response to the vaccine as demonstrated by increases in plasma CRP, after completion of the study, the manufacturer of the vaccine announced that several batches of its Salmonella typhi vaccine has been recalled due to concern that the vaccines may contain lower than expected antigen content. As such the negative findings concerning the impact of vaccination on measures of endothelial function are likely the result of a blunted inflammatory to the lot of vaccine which we administered.
2.6. Conclusion:

In conclusion, the present study was unable to demonstrate endothelial dysfunction after injection of *Salmonella typhi* vaccine in healthy volunteers. This is not consistent with previous papers who reported a blunted FMD after vaccination.\textsuperscript{27} The negative results observed in this study might be due to inadequate antigenecity of the vaccines used and our findings might not be relevant.
The effect of nebivolol on ischemia-reperfusion induced endothelial dysfunction

3.1. Abstract:

Objective: To determine if a single dose of 5mg nebivolol provides protection against IR-induced endothelial dysfunction in humans.

Background: IRI injury has been shown to cause abnormalities of endothelial function. Animal and human studies have demonstrated that nebivolol, a third generation beta-adrenergic blocker, increases the bioavailability of NO. Based on its pharmacologic effects, nebivolol may be an effective pharmacologic preconditioning agent, providing protection from IR injury.

Methods: In a double-blind, parallel design, 20 volunteers were enrolled. Baseline heart rate and blood pressure were obtained. Subsequently, subjects were randomized in a double-blind fashion to a single dose of oral nebivolol (5mg) or placebo. Twenty-four hours later, measurements of heart rate and blood pressure were repeated. Subsequently FMD of the radial artery was measured before and after IR (20 minutes of upper-arm ischemia followed by 20 minutes of reperfusion).

Results: There were no differences in heart rate, blood pressure or FMD between the two groups. IR significantly blunted FMD in the placebo group (FMD pre-IR: 8.0±2.4%; post-IR: 3.5±2.7%, P=0.005). However, there was no change in FMD following IR in those receiving nebivolol (FMD pre-IR: 6.7±3.2%; post-IR: 5.8±4.2%, P=NS versus nebivolol pre-IR).

Conclusion: The present findings demonstrate, for the first time in humans, that nebivolol prevents endothelial dysfunction induced by ischemia reperfusion. This was manifested by the ability of nebivolol to prevent the impairment of FMD after IR insult.
3.2. Introduction:
The main goal of treatment in patients with myocardial infarction is timely and effective reperfusion. Restoration of blood flow, however, is believed to contribute to myocardial damage, a phenomenon called ischemia-reperfusion (IR) injury.\textsuperscript{28,32} Importantly, endothelial cells are susceptible to IR injury and actively participate in this IR injury.\textsuperscript{40,152} The development of endothelial dysfunction caused by IR might prolong vasoconstriction after reperfusion and exacerbate tissue injury, leading to subsequent organ damage.\textsuperscript{26} The endothelium plays an important role in the response to IR injury and the development of endothelial dysfunction likely contributes to tissue injury caused by IR. As such, interventions protecting the endothelium from IR injury has important clinical relevance.

Animal\textsuperscript{153,154} and human\textsuperscript{26} studies have shown that IR injury is reduced by brief episodes of ischemia prior to prolonged ischemia, so called ischemic preconditioning (IPC). Importantly, studies have documented that some pharmacologic agents are able to induce a phenotype similar to that observed with IPC, a phenomenon termed pharmacologic preconditioning.\textsuperscript{155} These include sildenafil\textsuperscript{109} and GTN\textsuperscript{110} and a variety of other drugs. Although the biochemical pathways involved in early and late preconditioning (both ischemic and pharmacologic) are complex and incompletely understood, an increase in NO bioavailability appears to play a central role.

It has been suggested that the vasodilatory properties of nebivolol is mediated by increased production of the endothelium-derived relaxing factor, NO.\textsuperscript{144} In an in vitro study, Gao et al. demonstrated that nebivolol, a third generation beta-adrenergic blocker, has vasodilator effect that are inhibited by nitro-L-arginine, an inhibitor of eNOS, in canine conduit vessels.\textsuperscript{156} This finding was confirmed and extended by Cockcroft et al. who showed that nebivolol dilates human forearm resistance vessels by a mechanism that involves the L-arginine/NO pathway.\textsuperscript{157}

Present study was designed to examine whether nebivolol has the ability to protect the endothelium from the injury caused by IR. We hypothesized that nebivolol would be an effective pharmacologic preconditioning agent.

3.3. Materials and methods:
The Mount Sinai Research Ethics Board approved this study, and all subjects gave written, 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 at least 12 hours prior to the study. Exclusion criteria included any significant health problems, the use of medications (including supplemental vitamins), as well as risk factors for cardiovascular disease such
as hypertension, smoking, hypercholesterolemia, and/or a family history of premature cardiovascular disease.

Twenty healthy volunteers aged 18-30 years were recruited in a double-blind, randomized, placebo-controlled parallel trial. Baseline measurements included standing heart rate and blood pressure. Subjects were then randomized to receive a single oral dose of placebo or 5 mg of nebivolol. Twenty-four hours after study medication, standing heart rate and blood pressure measurements were repeated. Subsequently, radial artery FMD was measured as described above. After this measurement was completed, a pneumatic cuff placed above the elbow was inflated to 250 mmHg for 20 minutes to induce local ischemia. The cuff was then deflated followed by 20 minutes of reperfusion at which point FMD was measured again. Our laboratory has had significant experience in the use of this experimental protocol involving IR-induced endothelial dysfunction and the therapeutic impact of preconditioning.\textsuperscript{95,109} We elected not to test endothelium-independent vasodilators because our previous studies have demonstrated that this cycle of IR specifically impairs endothelium-dependent responses while leaving nonendothelium-dependent smooth muscle responsiveness unaltered.\textsuperscript{26} The dose of nebivolol chosen for this study was based on the finding that this dose has improving effects on endothelial function as evident by improvement in FMD after 4 weeks of treatment.\textsuperscript{164} One day interval from the drug dose to the measurements was chosen so the acute pharmacological effects of nebivolol would be separated from the downstream preconditioning effects.

\textbf{Figure 3.1}. Schematic representation of the experimental design of the study. Baseline blood pressure and heart rate were measured, after which subjects were randomized to a single dose of placebo or nebivolol capsule. Blood pressure, heart rate, and FMD/IR/FMD were performed 24 hours after randomization.

\textbf{3.3.1. Statistical analysis:}

Data are presented as mean ± SD. For our model of ischemic injury, sample size estimates were made based on previous data from our laboratory\textsuperscript{109}. IR decreased FMD responses from 7.9±3.3% to
1.2±2.3%. Prevention of 50% of this impairment by nebivolol required 10 subjects per group. This sample size yields a 1-β=0.8 and a 2-sided α of 0.05. 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:

There were no significant differences in resting blood pressure, resting radial artery diameter, baseline blood flow, reactive hyperemia, or pre-IR FMD between the two groups (Table 2.2. and Table 2.3.).

Table 2.1. Baseline characteristics of the study subjects.

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Nebivolol</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP (mmHg)</td>
<td>122±6</td>
<td>123±10</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>72±6</td>
<td>72±9</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>91±6</td>
<td>91±8</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>72±12</td>
<td>74±9</td>
</tr>
<tr>
<td>FMD (%)</td>
<td>8±2.4</td>
<td>6.7±3.2</td>
</tr>
</tbody>
</table>
Table 2.2. Arterial blood flow data. Blood flow is presented as mL/min.

<table>
<thead>
<tr>
<th></th>
<th>Pre-IR</th>
<th>Post-IR</th>
<th></th>
<th>Pre-IR</th>
<th>Post-IR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>After cuff deflation</td>
<td>Reactive hyperemia (%)</td>
<td>Baseline</td>
<td>After cuff deflation</td>
</tr>
<tr>
<td>Placebo</td>
<td>12.7±9.7</td>
<td>124.0±76.2</td>
<td>1455.3±1525.5</td>
<td>9.9±7.2</td>
<td>117.4±68.3</td>
</tr>
<tr>
<td>Nebivolol</td>
<td>14.7±12.1</td>
<td>119.5±33.9</td>
<td>1453.6±1192.3</td>
<td>12.8±11.2</td>
<td>121.8±41.5</td>
</tr>
</tbody>
</table>

Table 2.3. Arterial diameter data. Diameter is presented as cm.

<table>
<thead>
<tr>
<th></th>
<th>Pre-IR</th>
<th>Post-IR</th>
<th></th>
<th>Pre-IR</th>
<th>Post-IR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline diameter</td>
<td>Change in diameter after cuff deflation</td>
<td>Baseline diameter</td>
<td>Change in diameter after cuff deflation</td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>0.23±0.04</td>
<td>0.017±0.008</td>
<td>0.22±0.03</td>
<td>0.014±0.008</td>
<td></td>
</tr>
<tr>
<td>Nebivolol</td>
<td>0.26±0.03</td>
<td>0.019±0.014</td>
<td>0.25±0.03</td>
<td>0.01±0.007</td>
<td></td>
</tr>
</tbody>
</table>
Nebivolol decreased systolic blood pressure significantly 24 hours after its administration (Figure 3.2., Visit 1: 123±910 mmHg; Visit 2: 117±9 mmHg, P= 0.02). While in the placebo group, the decrease in systolic blood pressure was not significant (Figure 3.2., Visit 1: 122±6 mmHg; Visit 2: 120±7 mmHg, P=NS). Nebivolol treatment had no significant effect on either diastolic blood pressure (Figure 3.3) or heart rate (Figure 3.4).

**Figure 3.2.** Systolic blood pressure in placebo and nebivolol groups. In the nebivolol group, systolic blood pressure was reduced significantly 24 hours after nebivolol administration. Data are mean ± SD. *P=0.02 versus Visit1 nebivolol.

**Figure 3.3.** Diastolic blood pressure in both the placebo and nebivolol group. Diastolic blood pressure did not change significantly in either group.
IR significantly blunted FMD responses in subjects who received the placebo capsules (Figure 3.5., pre-IR: 8.0±2.4%; post-IR: 3.5±2.7%, $P=0.005$). In contrast, nebivolol administration prevented the impairment in FMD associated with IR (Figure 3.5., pre-IR: 6.73±3.24%; post-IR: 5.8±4.2%, $P=NS$ versus nebivolol pre-IR). Although the ANOVA revealed that there was no significant difference between the responses of the two groups, the group effect was of borderline significance ($P = 0.09$).
3.5. Discussion:

The present study was performed to examine whether the beta-blocker, nebivolol, is able to prevent endothelial cells from the dysfunction induced by ischemia-reperfusion. Nebivolol has been shown to have a neuroprotective effects during periods of spinal cord IR in rabbits. In this report, the authors hypothesized that this preconditioning effect was mediated by an antioxidant effect, with a decrease in free radicals produced in response to IR. A more recent study by Heeba et al. showed that pretreatment with nebivolol decreased iNOS expression while increasing eNOS expression in response to IR injury in a rat ischemic stroke model. This study also suggested that the pharmacologic preconditioning effect of nebivolol against IR injury was, in part, due to an antioxidant effect.

The present study demonstrates, for the first time in humans, the ability of the beta-blocker, nebivolol to create an endothelial pharmacological preconditioning effect in the setting of IR injury at the level of the conduit vasculature. Our results are consistent with evidence from animal models of ischemic injury, which have demonstrated the ability of nebivolol to decrease infarct volume and histopathological changes induced by IR. Although the observed protection in the nebivolol group appears quite robust, the ANOVA failed to reveal a significant difference when the responses in the placebo and nebivolol group. The group effect, however, was of borderline significance (P = 0.09) suggesting that this might represent a type II error.

Previous in vivo and in vitro studies have proposed that the vaso-relaxation induced by nebivolol is predominately endothelium-dependent and favorably affects the L-arginine/NO pathway. The exact mechanism by which nebivolol increases NO bioavailability is unclear, but the drug was shown to enhance the activity of phospholipase C, which increases the intracellular free calcium concentrations. Because the activity of eNOS is calcium/calmodulin-dependent, the increase in the concentration of intracellular free calcium leads to stimulation of this enzyme, which in turn enhances the synthesis of NO. As yet we have not done further studies to elucidate the mechanism of the observed pharmacologic preconditioning effects. Such studies could involve the concomitant administration of a NOS inhibitor and or an antioxidant intervention and will be the subject of future investigations.

3.5.1. Study limitations:

Limitations associated with the experimental model of IR in the human forearm need to be addressed. In contrast to animal models and clinical IR, it has been demonstrated that forearm model of transient
IR injury impairs endothelial vasodilatory responses while leaving the endothelium-independent responses unaltered.\textsuperscript{26} Thus, while we did observe a preservation of FMD following nebivolol administration, a direct protective effect of nebivolol on the underlying vascular smooth muscle cannot be inferred from these data. As such, the relevance of the findings of the present study to clinical IR, which is characterized by both vascular and tissue injury, is uncertain. Furthermore, although the impairment of FMD after IR is believed to be largely mediated by a dysfunctional endothelium\textsuperscript{161,162}, other factors such as sympathoexcitation may contribute to the altered FMD response in this model of IR. These factors are poorly understood and require further investigation.

3.6. Conclusion:
In summary, the present research demonstrates that IR produced a significant endothelial dysfunction in the control group that was observed as a significant blunting in FMD. This was consistent with previous studies. Nebivolol pre-treatment prevented the endothelial dysfunction produced by IR. Our results could have potential clinical implications. In view of the key role of endothelium-derived NO in the regulation of vascular tone, as well as on platelet function, it is likely that long-term endothelial dysfunction after reperfusion could lead to smooth muscle contraction and platelet adhesion and therefore lead to increased risk of vasospasm and thrombosis. Thus, the endothelial protection afforded by nebivolol administration could be associated with protection of endothelium against these adverse effects of reperfusion. To the best of our knowledge, these results represent the first human evidence of a direct endothelial pharmacological preconditioning effect by nebivolol.
The effect of nebivolol on the development of nitroglycerin-induced endothelial dysfunction and tolerance

4.1. Abstract:

**Objective:** To determine whether 5 mg/day nebivolol modifies the development of nitrate tolerance and endothelial dysfunction in subjects receiving continuous GTN (0.6 mg/hr) for seven days.

**Background:** Both experimental and clinical observations indicate that enhanced superoxide anion production is the underlying mechanism of nitrate tolerance. Recent observations in animal models have demonstrated that nebivolol, the third generation β-blocker with vasodilatory property, is able to inhibit vascular superoxide production.\(^{144,158}\) The proposed study was designed to investigate whether nebivolol prevents the development of nitrate-induced endothelial dysfunction and nitrate tolerance. The impact of GTN on endothelial function was assessed by intra-arterial infusions of Ach and the NOS inhibitor L-NMMA. The development of GTN tolerance was assessed using measures of systemic blood pressure and the FBF responses to intra-arterial GTN.

**Methods:** Healthy male volunteers were enrolled in this double-blind parallel study. Standing blood pressure and heart rate were obtained followed by measurement of baseline FBF by venous occlusion plethysmography. Subjects were then randomized in an investigator-blind fashion to receive continuous transdermal GTN (0.6mg/h) and placebo or transdermal GTN (0.6mg/h) and nebivolol (5mg/day), all for 7 days. On the last visit, which was 7 days after randomization, FBF was measured in response to incremental doses of ACh, L-NMMA, and GTN. **Note:** only 7 subjects have completed the study to date. The study was not completed because of time limitations.
4.2. Introduction:

Organic nitrates, such as GTN, are commonly used in the management of cardiovascular diseases including chronic congestive heart failure, acute myocardial infarction, and unstable angina.\textsuperscript{41,42} It is believed that the mechanism by which organic nitrates cause smooth muscle relaxation is via NO signaling pathway although the exact NO moiety mediating their effects remains uncertain.\textsuperscript{43} The development of tolerance, i.e. loss of hemodynamic and anti-anginal effects during continuous therapy, is a major factor limiting the efficacy of these drugs.\textsuperscript{41,42} Although the exact mechanism underlying the nitrate tolerance is poorly understood, a number of hypotheses have been proposed. One of the most important hypotheses is the free radical hypothesis.\textsuperscript{42} Both animal and human studies indicate that an important cause of nitrate tolerance is an increase in the vascular bioavailability of ROS. Evidence suggests that the main source of nitrate-induced ROS overproduction is the mitochondrial respiratory chain which leads to activation of vascular NADPH and a subsequent increase in NADPH oxidase-derived ROS.\textsuperscript{41} More recently, NOS dysfunction has also been implicated as a source of ROS production, and it seems that both of these enzyme systems contribute to enhanced ROS bioavailability during sustained GTN therapy. 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 ROS production as well as the investigation of several therapeutic strategies to prevent the nitrate-induced increase in ROS.\textsuperscript{144} Recent observations in humans have demonstrated that concurrent therapy with agents such as folic acid\textsuperscript{129} and atorvastatin\textsuperscript{103} can modify the development of tolerance associated with chronic GTN therapy. Importantly, the increase in NOS bioavailability and the development of NOS dysfunction during GTN (and other organic nitrate) therapy has also been shown to have adverse effects on endothelial function. Therefore, the increase in ROS bioavailability in response to organic nitrate therapy represents an explanation for two separate, but related phenomena. On the one hand they appear to explain the loss of nitrate hemodynamic and symptomatic effects over time. On the other they cause very significant abnormalities in endothelium-dependent vasomotor responses.

A study by Mollnau et al. examined whether nebivolol, a third generation β-blocker, can improve endothelial function by reducing vascular superoxide production and whether it can prevent eNOS uncoupling. Nebivolol was shown to have a potent inhibitory effect on vascular superoxide production in animal models of hypercholesterolemia, an effect that seems to be partly due to prevention of eNOS uncoupling. In addition, nebivolol was also shown to have inhibitory effect on NADPH oxidase.\textsuperscript{144} In another study authors investigated the protective effect of nebivolol against spinal cord IR and its
impact on IR-induced oxidative damage. This study confirms the antioxidant property of nebivolol as it prevented the increase in molandialdehyde (MDA), the marker of oxidative stress, after IR injury.\(^{158}\)

Whether co-administration of the \(\beta\)-blocker, nebivolol, can preserve GTN responsiveness and prevent the development of GTN-induced endothelial dysfunction in humans has yet to be established. We hypothesized that in healthy subjects, co-administration of nebivolol (5mg) with continuous GTN would prevent the development of nitrate-induced endothelial dysfunction and tolerance.

4.3. Materials and methods:
The Mount Sinai Research Ethics Board approved this study, and all subjects gave written, 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 at least 12 hours prior to the study. Study subjects were required to refrain from any form of exercise for at least a week 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.

Sample size calculation revealed that 24 subjects (12 each group) are required. Due to time constraints, this study was discontinued and only seven healthy volunteers aged 18-30 years were recruited in a double-blind, randomized, placebo-controlled parallel trial. After study admission, standing blood pressure and heart rate measurements were obtained using an automatic, calibrated sphygmomanometer (GE Healthcare, Mississauga, Ontario, Canada). Baseline FBF was measured by forearm venous-occlusion plethysmography as described below. Subjects were then randomized to 7 days treatment with placebo plus transdermal GTN patch (0.6mg/hr) or 5 mg of nebivolol plus transdermal GTN patch (0.6mg/hr). Subjects were asked to return to the laboratory for heart rate and blood pressure measurements 3, 24, and 72 hours after randomization. Seven days after randomization, standing heart rate and blood pressure measurements were repeated after which FBF was measured at baseline and in response to infused drugs. The endothelium-dependent vasodilator acetylcholine chloride (ACh, Novartis) was infused at 7.5, 15, and 30 \(\mu\)g/min. Following a washout period, GTN was infused at 11 and 22 nmol/min to test vascular reactivity to GTN. Finally, the NOS inhibitor L-NMMA (Bachem AG) was infused at 1, 2, and 4 \(\mu\)mol/min. The infusion rate was kept constant at 0.4 mL/min with a precision pump (Harvard apparatus). Each concentration was infused for 6 minutes, and FBF measurements were performed during the last 3 minutes. All responses were
evaluated as changes from a baseline value (normal saline infusion) immediately preceding each infused drug. Intra-arterial blood pressure was recorded after each infusion using the average of at least 15 cardiac cycles. ECG was monitored continuously. Between different drug infusions, normal saline was infused until the blood flow returned to baseline values. At the end of the study, the arterial line was removed, all study medications discontinued and the subjects were discharged from the laboratory.

4.3.1. Statistical analysis:
To calculate the number of subjects required to detect a significant change in FBF, we used previously reported values for healthy subjects after no treatment or prolonged exposure to GTN\textsuperscript{56,103}. This study required 12 subjects per group to see a 30% difference in FBF response. This sample size yields a 1-\(\beta=0.8\), with a 2-sided \(\alpha=0.05\).

**Figure 4.1.** Diagram of the experimental design of the study. Standing blood pressure (BP) and heart rate (HR) were obtained. Then baseline forearm blood flow (FBF) was measured. Subjects were then randomized to transdermal GTN patch (0.6mg/hr) with placebo or nebivolol (5mg/day) for 7 days. BP and HR measurements were repeated on day 2 and 4. Seven days after randomization, FBF was measured in response to ACh, GTN, and L-NMMA.

4.3.2. Plethysmography:
Subjects were asked to comfortably lay supine on a bed. Prior to the commencement of the study, the brachial artery of the non-dominant arm was cannulated under local anesthetic (1% lidocaine) using a 20-gauge plastic catheter (Cook, Bloomington IN).

Once cannulated, the catheter was 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 was 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 was
used to maintain a continuous infusion rate of 3 mL/hr in order to prevent thrombosis around the catheter.

Following arterial cannulation, both forearms were positioned above the level of the heart to allow for sufficient venous drainage during FBF measurement. Both upper-arms were supported with foam pads such that subjects were not elevating their arms under their own power. Cuffs were placed at the level of the upper-arms and wrists, as mentioned above (Upper arm: Hokanson 13 x 129 85 cm rapid version straight segmental cuff; Wrist: Hokanson 6.5 x 23 cm transmetatarsal cuff). Upper-arm cuffs were connected to a rapid cuff inflator (Hokanson), while wrist cuffs were connected to a manual sphygmomanometer. Upper-arm and wrist cuffs were 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) were placed on the widest part of both forearms and were connected to the plethysmograph (Hokanson). Once all equipment had been attached, subjects were allowed to rest quietly for a period of at least 20 minutes.

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

4.4. Discussion:

Organic nitrates are widely used in the management of cardiovascular diseases. Short-term administration of organic nitrates has hemodynamic effect, which rapidly diminishes during continuous therapy. This phenomenon, called nitrate tolerance, is the main factor limiting nitrate use. It is now believed that one of the most important causes of nitrate tolerance is increased vascular ROS bioavailability. Nebivolol was shown to have antioxidant property in animal models. Our aim in this study was to investigate whether nebivolol can prevent the development of endothelial dysfunction and tolerance induced by sustained GTN therapy. Unfortunately, this proposal could not be completed due to time constraints but we hypothesize that nebivolol has the ability to prevent nitrate-induced
tolerance and dysfunction. We believe that this study, if could be completed, would have an important clinical implication.
References


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