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NOVEL STRATEGIES TO IMPROVE METABOLIC PARAMETERS AND PRECONDITION DIABETIC HEARTS AGAINST ISCHEMIA/REPERFUSION INJURY

AMIT VARMA
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NOVEL STRATEGIES TO IMPROVE METABOLIC PARAMETERS AND
PRECONDITION DIABETIC HEARTS AGAINST ISCHEMIA/REPERFUSION INJURY

A dissertation submitted to the faculty in partial fulfillment of the requirements for the
degree of Doctor of Philosophy in Physiology and Biophysics

By

Amit Varma, MD

Doctor of Medicine, Virginia Commonwealth University, School of Medicine, 2006
Bachelor of Science, Virginia Commonwealth University, 2002

Major Director: Rakesh C. Kukreja, PhD
Department of Internal Medicine

Virginia Commonwealth University
Richmond, VA
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Acknowledgments

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<tr>
<td>ΔΨm</td>
<td>mitochondrial membrane potential</td>
</tr>
<tr>
<td>5-HD</td>
<td>5-hydroxydecanoate</td>
</tr>
<tr>
<td>ABCA1</td>
<td>ATP binding cassette A1</td>
</tr>
<tr>
<td>ADMA</td>
<td>asymmetric dimethylarginine</td>
</tr>
<tr>
<td>AGE</td>
<td>advanced glycation end products</td>
</tr>
<tr>
<td>AMI</td>
<td>acute myocardial infarction</td>
</tr>
<tr>
<td>AMPK</td>
<td>adenosine monophosphate (AMP) activated protein kinase</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein -1</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl2 associated X protein</td>
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<tr>
<td>Bcl2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BH₄</td>
<td>tetrahydrobiopterin</td>
</tr>
<tr>
<td>CAD</td>
<td>coronary artery disease</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>Cav-1</td>
<td>caveolin-1</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>CCL</td>
<td>chemokine (C-C motif) ligand</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CHF</td>
<td>congestive heart failure</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
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<tr>
<td>CREB</td>
<td>cAMP response element binding</td>
</tr>
<tr>
<td>CsA</td>
<td>cyclosporine</td>
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<tr>
<td>CSE</td>
<td>cystathione gamma lyase</td>
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CypD – cyclophilin D
CV – cardiovascular
DAG – diacylglycerol
DAP – diarylenypiperidone
DMSO – dimethylsulfoxide
ECG – electrocardiogram
ED – erectile dysfunction
eNOS – endothelial nitric oxide synthase
EPC – endothelial progenitor cells
ER – endoplasmic reticulum
ERK – extracellular signal related kinase
ET-1 – endothelin – 1
ETC – electron transport chain
FAD – flavin adenine dinucleotide
FFA – free fatty acids
FGF – fibroblast growth factor
FMN – flavin mononucleotide
GC – guanylate cyclase
GFAT - glutamine:fructose-6-phosphate amidotransferase
GLUT4 – glucose transporter 4
GPX – glutathione peroxidase
GRB2 – growth factor receptor bound protein 2
GSH – reduced glutathione
GSK-3β – glycogen synthase kinase-3β
GSSG – oxidized glutathione
GTP – guanosine triphosphate
H2S – hydrogen sulfide
HAT – histone acetyl transferase
HDL – high density lipoprotein
<table>
<thead>
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<th>Term</th>
<th>Description</th>
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<tbody>
<tr>
<td>HIF-1α – hypoxia inducible factor - 1α</td>
<td>LDH – lactate dehydrogenase</td>
</tr>
<tr>
<td>hs-CRP- high sensitivity c-reactive protein</td>
<td>LDL – low density lipoprotein</td>
</tr>
<tr>
<td>HSP – heat shock protein</td>
<td>LPL – lipoprotein lipase</td>
</tr>
<tr>
<td>ICM – ischemic cardiomyopathy</td>
<td>LPS - lipopolysaccharide</td>
</tr>
<tr>
<td>IHD – ischemic heart disease</td>
<td>LV – left ventricle</td>
</tr>
<tr>
<td>IKK-β - inhibitor of nuclear factor κ kinase β</td>
<td>MAPK – mitogen activated protein kinase</td>
</tr>
<tr>
<td>IL - interleukin</td>
<td>MCP – monocyte chemoattractant protein</td>
</tr>
<tr>
<td>iNOS – inducible nitric oxide synthase</td>
<td>MEK – MAPK kinase</td>
</tr>
<tr>
<td>IPC – ischemic preconditioning</td>
<td>MI – myocardial infarction</td>
</tr>
<tr>
<td>IPOC – ischemic postconditioning</td>
<td>MIP – macrophage inflammatory protein</td>
</tr>
<tr>
<td>I/R – ischemia/reperfusion</td>
<td>miR – microRNA</td>
</tr>
<tr>
<td>IRAG – inositol triphosphate receptor-associated cGMP-kinase substrate</td>
<td>mPTP – mitochondrial permeability transition pore</td>
</tr>
<tr>
<td>IRE1 – inositol requiring protein 1</td>
<td>mRNA – messenger ribonucleic acid</td>
</tr>
<tr>
<td>JNK – C-jun N-terminal kinase</td>
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</table>
mSOS – mammalian Son of Sevenless homolog
NADPH – nicotinamide adenine dinucleotide phosphate
NF-κB – nuclear factor kappa B
nNOS – neuronal nitric oxide synthase
NO – nitric oxide
NOS – nitric oxide synthase
NOX – NADPH oxidase
NTG – nitroglycerin
PAI-1 – plasminogen activator inhibitor -1
PANK – panthotenate kinase
PARP-1 – poly(ADP-ribose) polymerase-1
PCI – percutaneous coronary intervention
PDE – phosphodiesterase

PDK1 – pyruvate dehydrogenase
lipoamide kinase isoenzyme 1
PDX1 – pancreatic and duodenal homeobox-1
pGC – particulate guanylate cyclase
PGI₂ – prostacyclin
PI3K – phosphotidylinositol-3-kinase
PKA – protein kinase A
PKC – protein kinase C
PKG – protein kinase G
Prx – peroxiredoxin
PTP1B – phospho-tyrosine protein phosphatase 1B
RANTES – Regulated and normal T-cell expressed and secreted
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>RAGE</td>
<td>receptor for advanced glycation end products</td>
</tr>
<tr>
<td>RCT</td>
<td>reverse cholesterol transport</td>
</tr>
<tr>
<td>RhoK</td>
<td>Rho Kinase</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>sGC</td>
<td>soluble guanylate cyclase</td>
</tr>
<tr>
<td>SH2</td>
<td>Src-2 homology-2</td>
</tr>
<tr>
<td>SI/RO</td>
<td>simulated ischemia/reoxygenation</td>
</tr>
<tr>
<td>SIRT1</td>
<td>sirtuin1</td>
</tr>
<tr>
<td>SOCS</td>
<td>suppressor of cytokine signaling</td>
</tr>
<tr>
<td>SREBP</td>
<td>sterol regulatory element</td>
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<tr>
<td>binding protein</td>
<td></td>
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<tr>
<td>T3</td>
<td>tadalafil treatment for the type II diabetic trial</td>
</tr>
<tr>
<td>TAD</td>
<td>tadalafil</td>
</tr>
<tr>
<td>TGF-α</td>
<td>transforming growth factor α</td>
</tr>
<tr>
<td>TGF-β₁</td>
<td>transforming growth factor – β₁</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor – α</td>
</tr>
<tr>
<td>TTC</td>
<td>triphenyltetrazolium</td>
</tr>
<tr>
<td>TTE</td>
<td>transthoracic echocardiography</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>TXA₂</td>
<td>thromboxane A₂</td>
</tr>
<tr>
<td>UCP</td>
<td>uncoupling protein</td>
</tr>
<tr>
<td>UPR</td>
<td>unfolded protein response</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>VASP</td>
<td>vasodilatory stimulated phosphoprotein</td>
</tr>
<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low-density lipoprotein</td>
</tr>
<tr>
<td>VSMC</td>
<td>vascular smooth muscle cells</td>
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VEGF – vascular endothelial growth factor

WHO – World Health Organization

XBP-1 – X-box binding protein - 1
Insulin resistance and chronic hyperglycemia promote vascular damage, increase circulating levels of inflammatory cytokines and lead to increased morbidity and mortality. MicroRNAs (miRs) -103/107 have been shown to negatively regulate insulin sensitivity and glucose homeostasis. Based on complimentary binding profiles, the downstream target gene of miR-103/107 is caveolin-1 (Cav-1). We hypothesized that daily administration of the phosphodiesterase-5 inhibitor tadalafil (TAD) ± the
curcumin analogue (HO-3867) will attenuate inflammation, improve metabolic parameters and reduce infarct size after ischemia/reperfusion injury (IRI). Furthermore, we propose that TAD therapy will reduce myocardial expression of miR-103/107 and increase mRNA and protein levels of its target gene, Cav-1.

Leptin receptor null mice were randomized to receive daily injections of TAD (1mg/kg), HO-3867 (25mg/Kg), combination therapy, or control for 12 weeks with weight and fasting glucose monitored weekly. Upon completion, cardiomyocytes were isolated from each group and were subjected to simulated ischemia and reoxygenation (SI/RO) for cell viability and reactive oxygen species (ROS) measurement. Another set were subjected to IRI in a Langendorff model. Plasma samples were taken to measure plasma concentrations of cytokines. For miR expression, total RNA was isolated from TAD and DMSO treated mice and was subjected to reverse transcription and real time PCR using miR assay probes to determine expression.

TAD, HO-3867 and the combination of both attenuated fasting glucose levels, reduced myocardial infarct size after IRI and inflammatory cytokines when compared to control (p<0.05 for each vs. control). Cardiomyocytes isolated from each treatment groups and subjected to SI/RO demonstrated reduced necrosis as shown by trypan blue exclusion assay, ROS generation, and improved mitochondrial membrane potential as compared to DMSO (control). Likewise, both mRNA and protein expression of Cav-1 were reduced in diabetic hearts but were significantly increased in TAD treated diabetic
mice, which may be a mechanism to improve insulin signaling through downregulation of miR-103/107 and upregulation of Cav-1.

These studies suggest that TAD alone or in combination may be a unique strategy to improve metabolic parameters and precondition diabetic hearts against IRI.
CHAPTER 1

BACKGROUND AND SIGNIFICANCE

A. Diabetes and Heart disease

The prevalence of diabetes in the United States is increasing at an astronomical rate and the American Diabetes Association currently estimates that 8.3% of the population has diabetes and nearly 79 million people have insulin resistance and are currently at risk for developing diabetes.\(^1\) Accelerated vascular injury and inflammation are intimately associated with the complications of insulin resistance and diabetes and can lead to a number of micro- and macro-vascular insults such as retinopathy, nephropathy and painful neuropathy. Eventually more adverse complications result as the pathophysiologic consequence of the chronic hyperglycemic state. Hyperglycemia increases the expression of genes in the vascular smooth muscle cells (VSMC), tissue macrophages and endothelium, promoting the attraction, adhesion and subsequent transmigration of monocytes into the sub-endothelial space. In addition, the perpetual exposure of lipids and proteins to elevated glucose concentrations generates the formation of advanced glycation end products (AGEs), inducing reactive oxygen species (ROS), binding to cell surface receptors, inhibiting endothelial nitric oxide (NO)
production and increasing oxidative stress. The enhanced oxidative stress has several effects at the molecular and cellular level, which eventually contributes to macrovascular disease such as coronary heart disease (CHD). The oxidation of low-density lipoprotein (LDL) and glucose oxidation generate the formation of superoxide anions within the mitochondria, which generates hydroxyl radicals and nicotinamide adenine dinucleotide phosphate (NADPH) production within macrophages. This leads to significant endothelial dysfunction, impairing vasodilatation and promotes vascular injury, oxidative stress and AGE formation.

Unfortunately, data from clinical trials suggest that despite intensive control of hyperglycemia, the reduction of glycosylated hemoglobin levels to 6% does not prolong life or reduce vascular morbidity.\(^2\) This indicates that endothelial dysfunction is likely at the crux of the problem that leads to poor long-term outcomes and not the hyperglycemia itself. There is data to suggest that reduced levels of NO within the vascular endothelium contributes to impaired insulin utilization in patients with insulin resistance.\(^3\) Vascular NO is critical for normal vasodilatation and endothelial function and impairment of NO bioavailability and the NO-cyclic guanosine monophosphate (cGMP)--dependent protein kinase (PKG) signaling cascade is the central mediator of endothelial dysfunction.\(^4\) A number of clinical studies have shown that hyperglycemia and increased AGEs are key factors in potentiating vascular inflammation and increasing levels of ROS and oxidative stress.\(^5,6\)
The interconnected pathways in insulin resistance and heart disease reveal that endothelial dysfunction, insulin resistance, obesity, hyperglycemia, inflammation and oxidative stress are all interconnected with each perpetuating the next. This eventually leads to type II diabetes, which then further accentuates the vicious cycle. The common downstream event is the formation of atherosclerosis, peripheral vascular and coronary artery disease. This leads to the increased morbidity and mortality in this patient population.

This vascular milieu of elevated inflammation, impaired NO bioavailability, and oxidative stress plays an integral role in the progression of atherosclerosis and
subsequently acute coronary syndromes culminating in significant morbidity and mortality of the diabetic patient.\(^7\) (Figure 1).

Recent animal studies with NO synthase (NOS) inhibitors and genetically altered mice that lack the endothelial nitric oxide synthase (eNOS) gene, suggest that the NO signaling pathway may regulate and promote glucose uptake in myocytes.\(^8\)-\(^11\) Moreover, when mice lack the eNOS gene, they were unable to catalyze the formation of NO in the endothelium, had decreased oxygen consumption, increased weight gain and were found to be insulin resistant.\(^12\) In addition, several studies have indicated that insulin resistance itself may impair NO release and damage the endothelium through mechanisms that are reciprocally interconnected.\(^5,6\) For example, chronic hyperglycemia increases circulating levels of inflammatory cytokines, chemokines, and expression of intracellular adhesion molecule-1 (ICAM-1), hence contributing further to this pathognomonic state.\(^13\)

It was previously hypothesized that the vasodilatory action of a phosphodiesterase (PDE)-5 inhibitor would potentially release endogenous mediators from the endothelium such as bradykinin and adenosine and that this may trigger a signaling cascade that results in phosphorylation of eNOS and release of NO from the endothelium. The generation of NO activates soluble guanylate cyclase (sGC) resulting in enhanced formation of cGMP and subsequently downstream cGMP protein-dependent kinases. Within each cell, levels of cGMP are tightly regulated by PDEs, which cleave the 3’5’-cyclic-phosphate moiety of cGMP to produce the corresponding 5’
nucleotide. PDE-5 selectively hydrolyzes cGMP and its inhibition increases the bioavailability of cGMP. The PDE-5 inhibitors, including sildenafil (Viagra™), vardenafil (Levitra™), tadalafil (Cialis™) and avanafil (Stendra), have been approved by the Food and Drug Administration for the treatment of erectile dysfunction (ED). More recently, sildenafil and tadalafil (TAD) have been approved for the management of pulmonary arterial hypertension. TAD selectively inhibits PDE-5 hence increasing cGMP levels, however its effects can last up to 36 h, whereas the durations of action of sildenafil and vardenafil are generally 4 to 8 h. Moreover, TAD is a highly selective inhibitor of PDE with >10,000-fold selectivity for PDE-5 over PDE-1 to PDE-4 and approximately 700-fold selectivity for PDE-5 over PDE-6. TAD is also the only PDE-5 inhibitor whose activity is unaffected by food and has a relatively short time to onset of action (16–17 min).

Chronic administration of PDE-5 inhibitors has been associated with increased persistent vascular and endothelial function by increasing the level of endothelial cGMP generated by activation of eNOS. In streptozotocin-induced diabetic rats, long-term administration of the PDE-5 inhibitor DA-8159 prevented ED and preserved endothelial function. In a similar model, 14 days of treatment with sildenafil improved vasorelaxation through enhanced endogenous NO signaling. In addition, clinical studies have revealed a potential protective role of these compounds on endothelial function in short- and long-term assessments. In a large meta-analysis it was reported that endothelial dysfunction is a significant independent risk factor for cardiac death,
myocardial infarction (MI), stroke and the need for coronary revascularization. A study by Gazzaruso et al found that diabetic patients with ED were at increased risk for silent coronary artery disease and ED was a powerful predictor of cardiovascular (CV) morbidity and mortality. They went on to show that PDE-5 inhibitor use in type II diabetics was associated with a significant reduction of major adverse cardiac events. Likewise, a recent epidemiological study provided evidence of a strong correlation between the risk factors associated with metabolic syndrome (i.e. obesity, elevated fasting glucose levels, dyslipidemia, hypertension) and urinary cGMP excretion, suggesting that a reduction of NO bioactivity concurs with these CV risk factors. Interestingly, one study found genetic variations of the eNOS gene influenced energy expenditure, severity of glucose intolerance, and risk of developing type II diabetes. In a clinical trial, chronic (alternate-day) administration of tadalafil in men with ED had improved endothelial function as indicated by marked changes in serum markers of endothelial function, increased insulin levels and a robust decrease in the inflammatory marker, high sensitivity C-reactive protein (hs-CRP). Similarly, both acute and chronic administration of sildenafil improved endothelial function in patients with type II diabetes as observed by improved flow-mediated dilatation of the brachial artery. In this respect, the use of PDE-5 inhibitors are quite attractive for the long-term management of cardiovascular diseases.

A number of studies from our laboratory have shown that PDE-5 inhibitors including sildenafil have a powerful protective effect against myocardial
ischemia/reperfusion (I/R) injury, \textsuperscript{29-33} doxorubicin and post-MI heart failure. \textsuperscript{34-38} Mechanistically, we showed that sildenafil protects the heart against I/R injury through increased expression of inducible NOS (iNOS) / eNOS \textsuperscript{18, 39} activation of PKG, \textsuperscript{39, 40} phosphorylation and inactivation of glycogen synthase kinase-β (GSK-3β), \textsuperscript{40} opening of mitochondrial K$_{\text{ATP}}$ channels, \textsuperscript{29-31} and hydrogen sulfide (H$_2$S) generation. \textsuperscript{33} Insulin resistant and type II diabetic patients have decreased expression of eNOS, iNOS and impaired NO synthesis, therefore the chronic use of PDE-5 inhibitors may be potentially therapeutic because these compounds improve ED, at least in part, by correcting damage to the vascular endothelium by upregulating eNOS and iNOS and increasing NO levels. \textsuperscript{27, 28, 41}

Currently there is an imperative need for understanding the molecular and biochemical mechanisms underlying diabetic vascular complications and a multifaceted therapeutic strategy that targets the problem on many levels would be optimal. Therefore based on this background information, we propose that PDE-5 inhibitors would be ideal candidates to treat endothelial dysfunction, insulin resistance, and inflammation while protecting the diabetic heart against I/R injury.

According to WHO, ischemic heart disease (IHD) is the leading cause of morbidity and mortality in the world accounting for more than 7.2 million deaths each year and by the year 2030 over 26 million people will die from cardiovascular diseases. \textsuperscript{42} Prompt myocardial reperfusion of the infarct-related artery by either fibrinolysis or percutaneous coronary intervention (PCI) remains critical for reducing
infarct size. Recent advances in the field of cardiovascular medicine has led to new antplatelet and antithrombotic drugs and novel anti-proliferative drug-eluting stents which represent a significant step forward in improving long term outcomes in patients presenting with acute coronary syndromes. Unfortunately despite intense research in the field of molecular cardiology, we have no definitive intervention nor approved pharmacological agent indicated to completely eliminate or reduce myocardial damage induced by reperfusion injury—a condition where ischemic myocardium is subjected to sudden intracellular metabolic and biological changes upon flow restoration. With a monumental growth in the number of patients dying from IHD in both developed and underdeveloped countries, not only has it become imperative to reduce the time to reperfusion, but also to understand the fundamental pathological mechanisms of I/R injury so that safe and potent cardioprotective compounds can be developed that would render the myocardium resistant to reperfusion injury.

B. **Novel Pharmacological Agents in Cardioprotection and Insulin Resistance**

   i. *Phosphodieseterase-5 inhibitors*

   Phosphodiesterase-5 is an enzyme that catalyzes the degradation of cGMP and through inhibition with a PDE-5 inhibitor, leads to an increase in bioavailable cGMP. There have been 21 PDE genes identified and cloned, all of which can be further
classified into one of 11 PDE families based on homology and pharmacological and biochemical properties. PDEs are ubiquitous throughout the body and perform a number of functions. PDE-5 is the predominant enzyme in the corpus cavernosum, and plays a crucial role in penile erection. Likewise, immunohistochemical studies have demonstrated that PDE-5 is abundant in vascular and bronchial smooth muscle cells, renal tubules, lung tissue and platelets. Initially it was thought that PDE-5 was not expressed in the heart, but Shezaki et al provided the first evidence that PDE-5 was expressed in the canine myocardium and subsequently our laboratory showed its expression in the rodent heart. It is known that PDE-5 inhibitors facilitate an erection by increasing NO signaling by preventing enzymatic hydrolysis of cGMP in endothelial cells. The PDE-5 isoenzyme is widely expressed in the vasculature and is found in other vascular beds other than the penis. The efficacy of PDE-5 inhibitors is much lower in diabetic patients with ED compared to those without it and similarly the endothelial effectiveness of drugs that improve endothelial function is lower in diabetics than non-diabetics. However, it is quite possible that when part of a daily regimen, a PDE-5 inhibitor improves the responsiveness of dysfunctional endothelium and ameliorates inflammation—which would make this a novel strategy for preventing the development and progression of atherosclerosis and CV diseases in the diabetic patient.

In the last several decades, the overwhelming knowledge and understanding of the cardioprotective signaling pathways involved in protecting the heart from ischemic insult has laid the conceptual background for developing pharmacological agents that
target specific triggers and mediators which may provide similar effects. In 2002, our laboratory was the first to show that the PDE-5 inhibitor sildenafil could induce a “preconditioning effect,” in which pre-treatment with the drug protected the heart against subsequent I/R injury. For example, in a rabbit model of I/R, the Kukreja laboratory found that opening of the mitochondrial adenosine triphosphate (ATP) sensitive potassium channel (mitoK$_{ATP}$) was critical in the sildenafil-induced infarct-sparing effect. Using the mitoK$_{ATP}$ blocker, 5-hydroxydecanoate (5-HD), the cardioprotection could be abolished, hence confirming a downstream signaling role of the adenosine triphosphate (ATP) sensitive potassium channel. We confirmed the findings in the rodent model, in the isolated perfused heart model, and in the in vitro cardiomyocyte cell culture model in which isolated cardiomyocytes were subjected to simulated ischemia and reoxygenation (SI/RO). The results were consistent in that all PDE-5 inhibitors, whether sildenafil, vardenafil or tadalafil all had an infarct-sparing effect, could reduce cardiomyocyte necrosis and apoptosis, and on transthoracic echocardiography (TTE), could preserve post-ischemic left ventricular (LV) ejection fraction. Mechanistic studies revealed that the cardioprotective effects of PDE-5 inhibitors are largely dependent on: NO generation, opening of the mitoK$_{ATP}$ channel, activation of Protein Kinase C (PKC), adenosine A1 receptor, and through inhibition of PDE-5 with subsequent accumulation of cGMP and activation of downstream PKG-dependent signaling. This includes PKG-dependent phosphorylation of extracellular signal-regulated kinase (ERK)1/2 and GSK3β in conjunction with increasing B-cell lymphoma
protein (Bcl-2), which inhibits apoptosis through attenuating cytochrome c release and inhibiting opening of the mitochondrial permeability transition pore (mPTP)\textsuperscript{52} [Figure 2]. Lastly, PKG has its own independent effect on I/R injury as it too can open the mitoK\textsubscript{ATP} and limit infarct size through preserving ATP and decreasing the calcium (Ca\textsuperscript{2+}) influx into the mitochondria.\textsuperscript{53} In a rabbit model of I/R injury we compared sildenafil and vardenafil with nitroglycerin (NTG) to evaluate their cardioprotective effect when administered immediately at the time of reperfusion.\textsuperscript{54} Animals were subjected to 30 min of ischemia followed by 3 h of reperfusion with intravenous (i.v.) sildenafil, vardenafil or NTG being given for 65 min, beginning 5 min before reperfusion. We found both PDE-5 inhibitors had similar cardioprotective effects compared to control and the infarct-sparing effect was mediated through the mitoK\textsubscript{ATP} channel as the effect could be blocked by 5-HD. Surprisingly however, NTG failed to confer any cardioprotection. We hypothesized that this could be explained by either insufficient cGMP bioavailability relative to NO or a surge of free radicals at the time of reperfusion that could react with the NO derived from NTG forming peroxynitrite (ONOO\textsuperscript{-}) that then could lead to protein nitration and myocardial damage.\textsuperscript{54}

PDE-5 inhibitors have also been shown to prevent adverse cardiac remodeling in the experimental arena of ischemic cardiomyopathy (ICM). In 2008, we showed for the first time in a murine model of ICM, that chronic sildenafil treatment can attenuate LV dysfunction.\textsuperscript{49} Recent evidence suggests that Rho Kinase (RhoK), which is activated by
the small GTPase RhoA, plays an important role in the development of cardiovascular disease and pulmonary hypertension.

Figure 2. PDE-5 inhibitors and sGC activators: Mechanisms of action in cardioprotection.

Activation of soluble guanylate cyclase (sGC) by either nitric oxide (NO) or independently of an oxidized heme group within sGC by a sGC activator, leads to an increase in cyclic GMP (cGMP), and downstream protein kinase G (PKG). Cardioprotective effects by PKG are induced through opening of the mitochondrial $\text{K}_{\text{ATP}}$ channel and phosphorylation of glycogen synthase kinase (GSK)-3β, which prevents opening of the mitochondrial permeability transition pore (mPTP).
Moreover, RhoK functions as a signal transducer in actin cytoskeletal organization, gene expression, and muscular contraction. Non-selective inhibitors of RhoK reduce infarct size and adverse remodeling of the LV. Likewise, in two different models of pulmonary arterial hypertension, sildenafil inhibited RhoK and improved pulmonary pressures.\textsuperscript{55,56} Therefore, we evaluated if sildenafil treatment could attenuate the progression of heart failure and whether the signaling could be attributed to inhibition of the RhoA/ Rho-Kinase pathway.\textsuperscript{57} In our model of ICM, mice were subjected to permanent left anterior descending occlusion and 3 days post-infarction, when fractional shortening was less than 25\%, were started on either twice a day treatment with sildenafil or equivalent volume saline. Mice treated with sildenafil showed preservation of LV function and less end-diastolic dilatation compared to control at 7 and 28 days post-MI. Similarly, fibrosis and apoptosis were significantly reduced in the sildenafil treated group. Western blot analysis of sildenafil treated homogenates revealed enhanced Bcl-2: Bcl-2 associated X protein (Bax) ratio, that RhoK was significantly inhibited, and this was directly associated with PKG activation. Moreover, RhoK inhibition was PKG-dependent as use of the PKG inhibitor KT-5823 completely abolished any RhoK inhibition.\textsuperscript{57}

Today, more than 100 clinical trials with PDE-5 inhibitors have been completed or are currently ongoing focusing on the drugs' potential cardiovascular benefits.\textsuperscript{58} These potent agents have shown promising results in patients with diastolic heart failure
and reactive pulmonary hypertension and intense research is ongoing in patients with valvular and congenital heart disease, diabetes mellitus, and systolic heart failure.\(^{58}\) However, there have yet to be trials designed to evaluate its direct preconditioning effects. Future demonstration of the cardioprotective effects in patients with PDE-5 inhibitors could have a considerable impact on post-MI morbidity and mortality by bringing the phenomenon of pharmacologic preconditioning from the bench to the bedside.

\[\text{ii. Soluble Guanylate Cyclase Activators}\]

The gaseous ligand NO is an important signaling molecule involved in regulating a variety of biological and physiological processes. Intense research on NO in the past few decades has led to significant clinical evidence that reduced NO bioavailability or responsiveness to NO is a fundamental component in the pathogenesis of cardiovascular and endothelial diseases. NO is a potent vasodilator, which prevents platelet adhesion and aggregation and inhibits vascular smooth muscle proliferation.\(^{59}\) Currently there are a variety of cardiovascular diseases, including hypertension, IHD and congestive heart failure (CHF) that are treated by one of the many pharmacologic agents termed “NO-donors or nitrovasodilators.” These drugs mimic the action of endogenous NO by either bioconversion to NO and NO-associated compounds and activate its intracellular receptor sGC by nitrosylation of the heme moiety. This in turn,
increases by 200-fold the catalytic conversion of guanosine triphosphate (GTP) into the secondary messenger cGMP. Most often than not, the intracellular signal are then potentiated by activation of PKG. However, the use of these medications is quite limited secondary to the development of nitrate tolerance, insufficient bio-metabolism and non-specific interactions of NO, including peroxynitrite (ONOO\(^-\))-mediated tyrosine nitration. Furthermore, ROS within the vasculature reacts with NO and inhibits NO signaling, creating a state of NO resistance and decreased NO bioavailability in the endothelium. To avoid these disadvantages of nitrate therapy and oxidant stress, a new class of compounds have been recently discovered which can activate sGC independently of NO and may have a significant advantage over current NO-donor therapeutics.

Guanine nucleotidyl (guanylate) cyclases (GCs) are a widely distributed key signal transduction enzyme, which in response to various cellular stimuli, converts GTP to the secondary messenger cGMP. The transmembrane particulate GC (pGC) serves as a receptor for atrial (a-type), brain (b-type) and c-type natriuretic peptide and analogues of these peptides have become a mainstay for treating severely decompensated CHF. The cytosolic form of sGC is a heterodimer consisting of two \(\alpha\) and two \(\beta\) subunits with the \(\beta\) subunit having a prosthetic heme group. The heme moiety is positioned in the heme-binding domain of the \(\beta\) subunit and has a length of about 200 residues. The ferrous iron of the prosthetic heme group is coordinated
between four heme nitrogens and the axial ligand histidine-105 and the anchoring residues of the heme propionates tyrosine\textsuperscript{135}, serine\textsuperscript{137} and arginine\textsuperscript{139} all together constitute the heme-binding motif.\textsuperscript{61} NO can then bind to the heme and activate sGC, but only if the heme moiety is in its reduced form (ferrous state) (Figure 3). In pathological conditions of excessive oxidative stress, there is perpetual endothelial and vascular damage that also promotes platelet aggregation. In these conditions, there is an abundance of ROS and NO is rapidly scavenged by superoxide (O$_2^-$) to form ONOO$^-$ and the heme moiety of sGC is quickly oxidized from ferrous heme to ferric heme, rendering NO ineffective as its ligand—making conventional NO donor pharmacologic agents futile.\textsuperscript{60} This has led many scientists to search for a way to directly activate sGC and restore the NO-sGC-cGMP pathway, whether under conditions of reduced NO availability, or in pathological states where sGC is largely in the oxidized form.

The effects of cGMP are mediated by various cGMP effector systems such as cGMP-dependent protein kinases (PKG-1\textalpha, PKG-1\textbeta), cGMP-dependent ion channels (PKG-2) and PDEs.\textsuperscript{60} The substrates of PKG include inositol triphosphate receptor-associated cGMP-kinase substrate (IRAG) and recognition of the substrates by the PKG-1 isozymes is mediated by the NH$_2$-terminal leucine/isoleucine zipper (LZ) domain.\textsuperscript{62} Both PKG-1\textalpha and PKG-1\textbeta are derived from the same gene and differ only in the terminal NH$_2$ LZ domain. Overall, PKG-1 substrates fulfill various cellular functions
such as intracellular Ca$^{2+}$ and potassium concentration, Ca$^{2+}$ sensitivity, and organization of the intracellular cytoskeleton. PKG-2 substrates are involved in chloride transport, sodium/proton transport and transcriptional regulation. The degradation of cGMP is catalyzed by one of several PDEs and this led to the discovery of specific PDE inhibitors, notably the PDE-5 inhibitors. PDE-5 inhibitors have been successfully used to treat disorders of endothelial dysfunction such as erectile dysfunction and pulmonary hypertension while attempting to restore the NO-cGMP intracellular signaling by preventing cGMP breakdown$^{60}$ (figure 3).

Since in a majority of cardiovascular diseases such as atherosclerosis, hypercholesterolemia, and insulin resistance, insufficient NO-sGC-cGMP signaling and endothelial dysfunction is at the crux of the problem, two novel drug classes have recently been discovered—the sGC stimulators that can activate sGC independent of NO and the sGC activators that can activate sGC independent of NO and independent of the redox status of the prosthetic heme group.$^{60}$ Therefore, these compounds could potentially allow for restoration of the NO-sGC-cGMP signaling cascade in these pathological states—which would have otherwise been lost due to NO scavenging and/or oxidation of sGC.
Figure 3. Nitric oxide signaling pathway.

Illustration of the activation of sGC by nitric oxide, which increases cGMP and subsequently PKG formation. This leads to the favorable effects of vasodilatation, inhibition of apoptosis, platelet aggregation and inflammation. Likewise, the particulate guanylate cyclase (pGC) can be activated by one of the natriuretic peptides (atrial, b-type, c-type) and activate the same pathway producing the same downstream effects.
Oxidation of sGC makes NO unable to bind this receptor given the Ferrous to Ferric state of the heme-moiety and hence is unable to form cGMP. One of the newest sGC activators, BAY 58-2667, can bind independently of the heme-moiety, regardless of its oxidative state and still form cGMP and downstream PKG.

Soluble GC stimulators target sGC independent of NO, but still require the heme moiety to be effective. These drugs directly stimulate sGC activity and even enhance
the sensitivity of the reduced enzyme to low levels of NO bioavailability. In 1994, after
testing thousands of compounds, Ko and coworkers at Bayer Healthcare AG discovered
that an indazole derivative, YC-1, could independently activate sGC by 10-fold and
could synergistically activate sGC with submaximal NO up to 200-fold. YC-1 became
known as the first “NO sensitizer” and was a major breakthrough in the ability to restore
the NO signaling cascade even under conditions of decreased NO availability. YC-1 had
shifted the EC\textsubscript{50} for NO to the left by one order of magnitude and was shown to slow
down the deactivation of NO stimulated sGC. The next two compounds developed
were BAY 41-2272 and 41-8543, both of which were synthesized based on YC-1
structure. Both drugs have been shown to inhibit vascular smooth muscle proliferation
and platelet aggregation and produce significant vasorelaxation of the coronary arteries
and veins in rat hearts using a Langendorff isolated perfused heart model.

In 2002, after nearly a decade of research, scientists at Bayer identified a
completely new mechanism of enzyme activation, they had created a compound which
could not only activate sGC independent of NO, but could even activate it after removal
or oxidation of the heme and in fact, it preferred the oxidized or heme-free enzyme. This exciting new compound was called BAY 58-2667 and it was the first NO- and
heme-independent activator of sGC. BAY 58-2667 was found to activate sGC with EC\textsubscript{50}
and \(K_d\) in the extremely low nanomolar range, making it the most potent NO-
independent activator to date. In addition, removal or oxidation of the heme group only
seemed to potentiate sGC activation, making it quite the ideal compound for use in
cardiovascular diseases that are plagued with high oxidative stress, excessive amounts of ROS and low NO availability. Further studies into the mechanistic action of this new drug show that BAY 58-2667 is able to mimic the spatial structure of the sGC porphyrin ligand and therefore binds into the empty heme binding pocket or replaces an oxidized, weakly bound heme (figure 4). Therefore, these novel compounds which activate sGC independent of NO, the oxidation status or even presence of the heme moiety, represent a significant therapeutic potential for the treatment of various cardiovascular diseases.

In the past several years, a number of key animal studies have demonstrated the robust cardioprotective effects of sGC activators. In 2009 Korkmaz et al used a model of isoproterenol-induced MI after preconditioning rats with cinaciguat (BAY 58-2667) for 4 days and then subjected them to acute myocardial infarction (AMI). They then measured cardiac function by Millar catheter, gene expression, mRNA levels of transforming growth factor (TGF)-β and cyclooxygenase (COX)-2, and performed immunohistochemistry for cGMP and nitrotyrosine. They found cinaciguat treated rats had significantly higher cGMP levels in plasma, higher concentrations in the myocardium, decreased mRNA expression of COX-2 and TGF-β, and overall a lower mortality rate. During the post-infarction period, the treatment group had improved cardiac relaxation, contractility was restored to baseline, and there was no change in coronary blood flow after I/R, both of which were significantly reduced in the control group. Our lab recently evaluated the effects of cinaciguat on ischemic preconditioning
(IPC) and I/R injury by giving the drug prior to ischemia and immediately at the time of reperfusion in the rabbit and mouse model of AMI and then confirmed these findings in a primary adult cardiomyocyte cell culture model of SI/RO by determining the compound’s ability to reduce cell necrosis and apoptosis. We found that cinaciguat caused a 63 and 41% reduction in infarct size when given before I/R and at reperfusion in rabbits, respectively. In mice, cinaciguat pretreatment had an even more impressive 80% reduction vs. 63% when given before I/R compared to at the time of reperfusion. Treatment groups had preserved cardiac function as measured by TTE and likewise, cinaciguat reduced myocyte necrosis and apoptosis. Furthermore, using an inhibitor of the H₂S generating enzyme, cystathionine-γ-lyase (CSE), or an inhibitor of PKG, these infarct-sparing and functional improvements were lost thus providing evidence that cinaciguat likely induces protection through PKG-mediated H₂S generation. Recently, Radovitz et al used a canine model of hypothermic cardioplegic arrest and extracorporeal circulation of coronary artery bypass surgery (CABG) to evaluate if cinaciguat could precondition the myocardium and effect endothelial function. They found that treatment with a sGC activator led to higher myocardial ATP levels, improved contractility, improved coronary blood flow and endothelial function.

Even though sGC activators have yet to be pursued for their infarct-sparing effects in the patient setting, ongoing research in various in vivo and in vitro models has been promising. However, given the recent termination of several major randomized controlled trials including: COMPOSE 1, COMPOSE 2 and COMPOSE EARLY using
cinaciguat for the treatment of congestive heart failure, it may be too early to explore clinical therapeutic options until the drug is thoroughly studied in various animal models.\textsuperscript{71} Conversely, the oral sGC activator Riociguat (BAY 63-2521), is actively being evaluated for the treatment of patients with pulmonary hypertension and thus far seems to be much more promising with at least a dozen clinical trials currently underway\textsuperscript{72}.

\textit{iii. Curcumin}

Turmeric (\textit{curcuma longa}) has been used by those practicing “Ayurvedic medicine” since its early discovery in 3000 b.c. in order to treat a variety of inflammatory conditions and ailments including obesity. Curcumin (diferuloylmethane) is the yellow-pigment, beta-diketone constituent of the spice turmeric derived from the rhizome of the \textit{curcuma long} plant (Figure 5). It is a natural polyphenolic phytochemical compound that is cell-permeable and known to possess histone acetyltransferase (HAT) inhibitory activity with specificity for p300/cAMP response element binding protein (CBP).\textsuperscript{73} It has a diverse range of molecular targets; including transcription factors, cytokines, and enzymes and as a result it has potential anti-inflammatory, anti-oxidant, anti-thrombotic, and cardiovascular protective effects. In experimental studies, it has been shown to lower plasma cholesterol, fasting glucose levels and increases hepatic glycogen levels.\textsuperscript{74} Likewise, through inhibiting p300/CBP it ameliorates post-infarction LV remodeling and by activating pro-survival kinases, limits infarct size.\textsuperscript{75-77} However, one
major caveat is that it has poor bioavailability and potency. Recently, a novel class of curcumin analogues have been synthesized, namely the diarylidenylpiperidones (DAPs), which have been chemically created to elude this problem. By incorporation of a piperidone ring in the β-diketone backbone structure and fluorinating the phenyl group, 4 DAPs have been synthesized by the Kuppusamy group—H-4073, HO-3867, HO-4318 and HO-4200. Of these compounds, HO-3867 (3,5-bis(4-fluorobenzylidene)-1-[(2,2,5,5-tetramethyl-2,5-dihydro-1-hydroxy-pyrrol-3-yl) methyl]piperidin-4-one), has been shown to be promising in a number of cancer studies, and a recent study has shown it to be cardioprotective against doxorubicin-induced cardiotoxicity. In general, when compared to curcumin, the DAP compounds are significantly more effective and exhibit potent anticancer efficacy in vitro against breast, colon and ovarian cancers.

The first report to show that curcumin could lower blood glucose in human diabetic subjects dates back to 1972 and since then, more than 3000 papers have been written on its effect on obesity and obesity-related complications. Likewise, numerous recent publications have confirmed that treatment with curcumin can modulate targets involved in metabolic diseases and prevent high-fat diet-induced diabetes in rat models and genetically induced diabetes in both mouse and rat models.
From a mechanistic perspective, these beneficial effects are likely afforded by attenuation of insulin and leptin resistance, amelioration of inflammatory cytokine expression and by increasing fatty acid oxidation and antioxidant enzyme levels. One recent study showed that curcumin-treated diabetic rats had lower blood glucose levels and glycosylated hemoglobin levels in addition to oxidative stress levels. When looking at other metabolic parameters such as cholesterol in diabetic animals, dietary curcumin significantly lowered blood triglycerides, cholesterol, and inhibited lipid peroxidation in liver microsomes and mitochondria.
C. Ischemia/Reperfusion Injury

Numerous experimental studies over the past 25 years have demonstrated the importance of the mPTP as a critical determinant of cardiomyocyte injury and necrosis after coronary I/R. Under normal physiologic and ischemic conditions, the mPTP is closed and the inner mitochondrial membrane remains impermeable to nearly all ions and metabolites. However during reperfusion, Ca\(^{2+}\) overload and ROS accumulation within the mitochondria leads to formation of transition pores allowing molecules up to 1.5 kDa to equilibrate across the membrane. Upon permeability transition, mitochondria lose their ability to maintain a pH gradient and produce ATP. This leads to the loss of ionic and metabolic homeostasis. Similarly, due to newly created osmotic forces, the mitochondrial matrix swells and as a result, the outer membrane ruptures causing cytochrome c and other proapoptotic factors to be released into the cytosol. Accordingly, opening of this inner mitochondrial membrane transition pore is strongly linked with lethal reperfusion injury which ultimately leads to cardiomyocyte necrosis and apoptosis. Griffiths et al demonstrated that opening of the mPTP does not occur during periods of non-lethal ischemia, but rather during the first few minutes of reperfusion. The opening of transition pore actually coincided with the rapid correction of the acidosis which occurred during the ischemic phase (figure 5).

The significance of the mPTP in I/R-induced cell death was first recognized by Hausenloy and Yellon with the use of the mPTP inhibitor—cyclosporine A (CsA).
Despite the powerful immunosuppressive effects of CsA, it has potent inhibitory effects on the transition pore through modulating cyclophilin D (CypD), a key molecular component of the mPTP. Under the presence of high matrix Ca\textsuperscript{2+} concentrations, during reperfusion the CypD can modify the conformation of the inner mitochondrial membrane allowing for the formation of a large pore/channel and creating significant permeability transition.\textsuperscript{87, 92} Hausenloy \textit{et al} demonstrated that CsA significantly reduced infarct size when given at the time of reperfusion in an isolated rat heart model.\textsuperscript{91} Similarly, using an \textit{in vivo} rabbit model, a pharmacological ischemic postconditioning (IPOC) effect was demonstrated when CsA was given 1 min prior to reperfusion and a 50% reduction in infarct size was obtained.\textsuperscript{93} Additionally, the role of CypD-dependent mPTP in IPOC has been evaluated using transgenic mice lacking CypD.\textsuperscript{94} These mice developed smaller infarcts and could not be post-conditioned. This further suggests that lethal I/R injury is mediated by Ca\textsuperscript{2+} overload, conformational change of the transition pore by CypD and subsequent pore opening.

D. \textit{Ischemic Preconditioning and Cardioprotection}

The search for infarct-sparing treatment spans nearly four decades when in 1971 Braunwald and colleagues introduced a novel concept: measures designed to improve coronary perfusion or reduce oxygen demand as late as 3 h after an acute thrombosis might limit infarct size.\textsuperscript{95}
Figure 6. Reperfusion injury and mitochondrial-induced ROS production and apoptosis.

During reperfusion, there is an acute overload of Ca$^{2+}$ ions within the cell as the cell attempts to compensate for the build-up of H$^+$ ions during the ischemic phase. This triggers formation transition pores and increases ROS formation. This leads to loss of the mitochondrial membrane potential and cytochrome $c$, which triggers activation of apoptosis.

Over the next 15 years, angioplasty became the only reperfusion therapy proven successful at limiting infarct size despite intense pre-clinical research with a number of
pharmacological agents in a variety of animal models. It wasn’t until 1986 when Murry et al discovered the phenomenon of IPC in a canine model of I/R.\textsuperscript{96} They discovered that several brief periods of left circumflex coronary artery occlusion prior to prolonged ischemia reduced infarct size by 75\% when compared to canines subjected only to the prolonged ischemia.\textsuperscript{96} These brief periods of sublethal ischemia induced protective signaling mechanisms by which the myocardium became preconditioned to the subsequent ischemic insult. Interestingly years leading up to the discovery of IPC, clinicians noticed that patients with CHD, particular those presenting with unstable angina or acute AMI and had at least one or more prior episodes of angina had less chest pain, less ST-segment deviation on electrocardiogram (ECG), and smaller infarct sizes despite an overall increase in ischemic time.\textsuperscript{97} This clinical paradox became aptly termed the “warm-up phenomenon.” The exact etiology is still unknown, but one of the speculated mechanisms is thought to be through triggered activation of the preconditioning signaling pathways in the “at risk” myocardium.

The protective effects of IPC occur in two distinct phases: an “early phase” that rapidly develops after an ischemic insult and dissipates within 2-3 h and a “delayed phase” which reappears about 24 h and persists for nearly 72 h.\textsuperscript{98} The major difference between these two phases is that the early phase is effective at limiting I/R injury and less effective at preventing post-infarction myocardial “stunning” or contractile dysfunction.\textsuperscript{99} From a cellular level, early IPC results in activation of transcription factors and results in compartmentalization, modification and translocation of existing
molecules and proteins. The delayed phase is less effective at limiting infarct size but helps dampen ischemia-induced stunning. Furthermore, in this delayed phase the cardioprotective effects are largely exerted by newly synthesized proteins that were activated for *de novo* synthesis during the early phase.

In 1991 Downey and colleagues discovered that IPC was in fact a receptor-mediated phenomenon by reporting the adenosine A1 receptor acted as a trigger to protect the rabbit heart. Further research led to the conclusion that activation of any G-coupled receptor can trigger a preconditioned state and can work in parallel. Interestingly, blockade of one of these receptor types does not abrogate IPC but rather increases the threshold needed to trigger the preconditioning. Therefore adenosine and other endogenous autacoids such as opioids and bradykinin, all activate their respective receptor G-protein coupled receptor and trigger downstream cardioprotective signaling pathways which include: ERK1/2, phosphatidylinositol-3-kinase (PI3K)/Akt, PKC and PKG which can ultimately lead to the phosphorylation and inactivation of GSK3β. This inactivated form of GSK3β inhibits the opening of the mPTP, which plays a critical role in cardiomyocyte death. Other key mediators of cardioprotection are the ATP-sensitive potassium channels of which there are two types—the sarcolemmal (surface KATP channel) and mitochondrial (mitoKATP channel). First described in cardiac ventricular myocytes, their significance in preconditioning was initially shown by Gross and colleagues. Pharmacological mimetics that could open the KATP channel would protect the myocardium, whereas blockers of the channel abolished the
cardioprotection. Subsequently, it was found that the mitoK\textsubscript{ATP} channels played a more significant role in IPC than the surface channels. However as years of research carried on, we learned both channel types likely have some role in preconditioning. In fact, the most convincing data demonstrates that transgenic mice without functioning sarcolemmal K\textsubscript{ATP} channels could not be preconditioned despite having completely intact mitoK\textsubscript{ATP} channels.\textsuperscript{104}

E.  \textit{Endothelial dysfunction}

Vascular endothelial cells form the vascular endothelial layer and healthy cells play an important physiological role.\textsuperscript{105} They respond to maintain vascular integrity, regulate vascular tone and permeability, vessel wall inflammation and thromboresistance.\textsuperscript{106} Likewise, they can secrete a variety of vasoactive substances including vasodilators such as NO, prostacyclin (PGI\textsubscript{2}), and endothelium-derived hyperpolarizing factor along with vasoconstrictors such as endothelin-1 (ET-1) and thromboxane A\textsubscript{2} (TXA\textsubscript{2}).\textsuperscript{107} Endothelial dysfunction is characterized by various functional alterations in the vascular endothelium that include an imbalance in the release of vasoactive substances, that can lead to thrombosis, vasospasm or impaired vasodilator responses; enhanced ROS generation, inflammation, apoptosis and adverse remodeling.\textsuperscript{107} Several mechanisms are implicated in the pathogenesis of endothelial dysfunction of which impaired NO bioavailability plays a pivotal role and is likely the
result of reduced NO production and its increased inactivation by ROS.\textsuperscript{107} Although considered the earliest marker of impaired vascular health, endothelial dysfunction is initially asymptomatic and it often precedes the development of atherosclerosis.\textsuperscript{106} In the insulin resistant patient, the endothelium is exposed to hyperglycemic conditions that activates an apoptotic process and leads to intimal denudation. Initially $\beta_1$-integrin cascade acts as the initial sensor for programmed cell death and initiates the apoptotic process.\textsuperscript{107} Upon integrin activation, p38 mitogen activated protein kinase (MAPK) and C-jun N-terminal kinase (JNK) are activated downstream, which lead to endothelial cell apoptosis. Likewise, flow stress can also initiate apoptosis through activation of the endoplasmic reticulum (ER) stress response and downregulation of vascular endothelial-cadherin, both of which activate caspase proteins.\textsuperscript{107} The end result is loss and detachment of endothelial cells into the plasma. Recently McClung \textit{et al} have shown that diabetic patients, irrespective of glucose control, have significantly higher levels of circulating endothelial cells.\textsuperscript{108} Moreover, it has been shown that endothelial derived microparticle levels are predictive of coronary artery lesions, lipid levels, hypertension, and duration of diabetes.\textsuperscript{109} Accordingly, as endothelial cells have limited ability for self-repair with low proliferative potential, an effective means of endothelial repair is necessary to re-establish vessel integrity and attenuate the pro-atherosclerotic process.\textsuperscript{107}

Endothelial repair is accomplished through the contribution of circulating endothelial progenitor cells (EPCs) in both physiological and pathological conditions.
EPCs are immature cells that can differentiate into mature endothelial cells and play a critical role in endothelial homeostasis and cardiovascular health. Recent data show that many different risk factors for CHD directly correlate with circulating EPCs and that higher EPCs portend a decrease in CV morbidity and mortality. Diabetic patients have less circulating EPCs than healthy controls and the progenitor cells they have display functional impairment such as reduced proliferation, adhesion, migration and incorporation into tubular structures. One explanation is the increased oxidative stress in diabetic patients that interferes with the interaction between the vascular wall and normal EPCs. In addition, angiogenic factors such as vascular endothelial growth factor (VEGF) and hypoxia inducible factor-1α (HIF-1α) are reduced in the diabetic patient whereas diabetic animal models have shown reduced mobilization of EPCs from the bone marrow after myocardial I/R injury. This data was supported as HIF-1α upregulation improved post-ischemic neovascularization in diabetes. Likewise, in an in vitro model, hyperglycemia through the production of ROS impaired proliferation, survival, and function of cultured EPCs with a concomitant decrease in NO production, however this could be corrected with insulin therapy.

It is now well accepted that endothelial dysfunction is part of an interconnected pathological process of which long term vascular complications in the diabetic patient are often fatal. In the hyperglycemic setting, both reduced NO bioavailability and increased oxidative stress are key determinants compromising vascular health. Therefore therapeutics designed to improve both parameters would be an ideal dual
strategy. In addition, there is a remarkable amount of literature supporting EPCs central role in the development and progression of diabetic complications and data suggest the metabolic interventions that improve glucose homeostasis may also be able to improve vascular biology and EPC levels.

i. *Nitric oxide pathway and its dysfunction*

Nitric oxide which has long been known as endothelium-derived relaxing factor was discovered in 1980s by Nobel laureates Ferid Murad, Louis Ignarro and Robert Gurchgott.\(^\text{114}\) Initially deemed a toxic air pollutant and pro-oxidant mediator, novel experiments and extensive studies carried out revealed NO to be one of the most ubiquitous biological substances within our physiological system. NO is known for its vital regulatory roles in vascular and metabolic health and in 1992 it was deemed “molecule of the year” by *Science*.\(^\text{115}\) Apart from its potent vasodilatory effects, it has powerful and important anti-thrombotic, anti-proliferative and anti-inflammatory features and today nitrate donor therapy is used for acute angina, CHF and pulmonary hypertension.\(^\text{116}\)

Nitric oxide is produced by many different tissues through a five-step oxidation process of L-arginine to NO and L-citrulline by one of the 4 isoforms of NOS (iNOS, eNOS, neuronal NOS and mitochondrial NOS).\(^\text{117}\) The production of NO via eNOS can be manifested through two different pathways—either a receptor-dependent mechanism
or receptor-independent pathway which leads to elevated intracellular Ca\(^{2+}\) levels and Ca\(^{2+}\)-calmodulin dependent activation of eNOS.\(^{118,119}\) Additionally, eNOS activity is increased upon phosphorylation at serine\(^{1177}\) mediated by the Akt pathway. More importantly, under basal conditions eNOS is kept in a tonic “inhibitory” state by being bound to caveolin-1 (Cav-1). Upon activation by stimuli, eNOS dissociates from Cav-1 and efficient NO production occurs. Conversely, iNOS-mediated NO production can occur independent of a Ca\(^{2+}\)-calmodulin mechanism and furthermore, iNOS expression has been shown to be upregulated in a number of cardiovascular diseases. Each NOS isoenzyme consists of two different domains, an oxygenase and reductase domain and coupling of the two domains is required for proper NO production.\(^{117}\) For NO synthesis, a number of co-factors are required such as \(\text{O}_2\), nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (BH\(_4\)), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN).\(^{118,120}\) Following release from an endothelial cell, NO diffuses to vascular smooth muscle where it can bind to sGC and it can increase cGMP levels. This induces smooth muscle relaxation, vasodilatation, and increases blood flow.\(^{53}\) In conditions where NOS becomes “uncoupled,” thereby becoming a monomer, molecular \(\text{O}_2\), as opposed to L-arginine, becomes the substrate for the NOS, which generates superoxide (\(\text{O}_2^-\)) anion instead of NO and increases cellular oxidant stress.\(^{117}\)
Nitric oxide is a highly lipophilic free radical with a short half-life, readily forms nitrogen oxides, and is extremely reactive which limit its bioavailability. However, NO-containing compounds can serve as reservoirs of bioactive NO and thus can not only participate in NO-related reactions, but also travel to remote tissue via the circulation.\(^{117}\) NO signaling occurs via one of 3 different mechanisms—via sGC activation, S-nitrosylation in which NO covalently and reversibly forms S-nitrosothiol groups with reactive cysteine thiols, and through formation of peroxynitrite, can activate MAPKs. Most of its effects are mediated through S-nitrosylation in a cGMP-independent mechanism.\(^{121}\)

The most important determinant of NO generation is blood flow, shear stress and pulsatile stretch.\(^{122}\) The laminar shear stress and dragging force exerted by fluid over the endothelial surface is a critical stimuli for endothelial NO release. Acutely, this leads to activation of eNOS and NO release for vasodilatation, which reduces the shear stress and chronically upregulates eNOS expression.\(^{122}\) In contrast low shear stress, turbulent blood flow, local shear gradients, stasis of flow, and rapidly changing flow disrupt endothelial cell function and endothelial derived NO production.\(^{117}\) In addition, NO acts as a counter measure against basal vasoconstrictors such as sympathetics, angiotensin II and ET-1.\(^{123}\) Angiotensin II potently stimulates vascular and leucocyte nicotinomide adenine dinucleotide (NADH) /NADPH and PDE thereby increasing cGMP hydrolysis and attenuating PKG-dependent kinase activity. Likewise, ET-1 is not only a potent vasoconstrictor, but is highly inflammatory, stimulates endothelial NADH/NADPH, which
increases oxidant stress and decreases vascular NO production via ET-A receptor binding.\textsuperscript{117}

The impairment of NO bioavailability has been implicated in a number of cardiovascular diseases including pathophysiological processes associated with insulin resistance, hyperlipidemia and hypertension. Under physiological conditions, insulin stimulates NO production in endothelial cells, and insulin resistance disturbs intracellular signal transduction, which results in an attenuated PI3K-Akt cascade, decreased eNOS activation and subsequently NO bioavailability.\textsuperscript{117} In fact, Insulin sensitivity is enhanced directly through NO-cGMP-PKG signaling through inhibition of the small GTPase Rho/Rho Kinase with insulin-receptor substrate-1 (IRS-1). In normal conditions, NO reacts and scavenges O$_2^-$ anion, thus effectively protecting the vascular tissue from oxidative stress and dampening the inflammatory response.

Both impaired NO bioavailability and dysfunctional NOS link vascular and metabolic disease and represent an early link between increased cardiovascular mortality in the diabetic patient. One theory for the overall decrease in NO in this patient population is the increased levels of the endogenous NOS inhibitor asymmetric dimethylarginine (ADMA).\textsuperscript{124} Similarly, given the overall increased oxidative stress in diabetics, BH$_4$ is oxidized which uncouples NOS and leads to even higher levels of O$_2^-$ anion and reduces NO production by NOS.\textsuperscript{125} It has also been demonstrated that diabetic patients that have had an \textit{i.v.} infusion of L-arginine had lower levels of NO
formation than did normal controls.\textsuperscript{126} In addition to the reduced bioavailability of NO, the diabetic milieu impairs the anti-proliferative properties of NO.\textsuperscript{120} This occurs primarily through cGMP-dependent and independent pathways. In the cGMP dependent manner, activation of cGMP also leads to an increase in cyclic adenosine monophosphate (cAMP) and its downstream effector, protein kinase A (PKA) which results in reduced intracellular Ca\textsuperscript{2+} flux and attenuation of the proliferative MAPK cascade.\textsuperscript{120} In the cGMP independent pathway, NO indirectly limits polyamine formation necessary for DNA synthesis by inhibiting arginase and ornithine decarboxylase while it also upregulates Fas expression in VSMC which induces apoptosis.\textsuperscript{120} With the attenuated NO bioavailability, this pathway is interrupted and leads to VSMC hypertrophy. Furthermore, VSMC proliferation has been linked to augmented stimulation of the MAPK pathway by insulin receptor activation.\textsuperscript{127}

Nitric oxide also has potent anti-thrombogenic properties, can be released from the endothelium and platelets, limits activation and aggregation of platelets through inhibition of the PI3K cascade, and suppresses platelet expression of adhesion molecules.\textsuperscript{128} Similarly, NO can react with O\textsubscript{2}\textsuperscript{−} anion to form ONOO\textsuperscript{−}, which can block production of the thrombogenic metabolite TXA\textsubscript{2}. Moreover, NO regulates and inhibits the upregulation of adhesion molecules on endothelial cells which would otherwise lead to leucocyte recruitment, adhesion, and migration and the initiation of plaque formation.\textsuperscript{120} The diabetic is known to have upregulated adhesion molecule expression,
circulating inflammatory cytokine levels with enhanced response to vascular injury primarily associated with enhanced insulin signaling through MAPK, as this pathway has also been shown to increase adhesion molecule expression.\textsuperscript{129}

F. \textit{Insulin and Insulin resistance}

Insulin is a key hormone with a critical role in growth and development of various tissues and for the homeostasis of plasma glucose levels. It is secreted as pre-proinsulin by the $\beta$-pancreatic cells as an inactive single precursor that directs its passage into vesicle cells.\textsuperscript{130} The proteolytic removal of the signal sequence results in the formation of pro-insulin. During periods of elevated plasma glucose levels and increased amino acid concentration, pro-insulin is secreted and converted to its active form, insulin, by various proteases.\textsuperscript{131} The active insulin molecule consists of an A and B chain that are held together by two disulfide bonds.\textsuperscript{131} The primary function of insulin is to stimulate glucose uptake into skeletal muscle and adipose tissue through increasing glucose transport, while reducing hepatic glucose production (via glycogenolysis and gluconeogenesis). Furthermore, it regulates lipid metabolism by inhibiting lipolysis within adipose tissue and increasing hepatic lipid synthesis.\textsuperscript{130, 131}

Type II diabetes is a complex disease state resulting from altered insulin secretion combined with the resistance of specific target tissue to the effects of insulin, most notably liver, adipose tissue, and skeletal muscle. Insulin resistance was first described in 1936 by Harold Himsworth, a preeminent clinician who concluded that
diabetes could be distinguished into two different types—insulin-sensitive which appears to be caused by a deficiency in insulin levels, and the insulin-insensitive type, which was not due to any deficiency in circulating insulin levels. Today, insulin sensitivity is synonymous with the ability of insulin to mediate disposal of an infused glucose load and the more efficient this process, the more insulin sensitive the person. Therefore, the inefficient person is deemed to be insulin insensitive and to maintain normal or near-normal glucose levels, the body must secrete higher than normal levels of insulin to compensate—a state of hyperinsulinemia. In 1988, it became evident that patients with compensatory hyperinsulinemia had associated complications and that this state contributed significantly to the development of CHD. Likewise, it was shown that many other abnormalities were persistently associated with insulin resistance in addition to glucose intolerance and hyperinsulinemia, such as decreased HDL, increased plasma triglyceride concentrations and hypertension. This cluster of pathologies became known as metabolic syndrome X and was associated with a significant risk of CHD. In adipose tissue, insulin decreases lipolysis thereby reducing free-fatty acid (FFA) efflux from adipocytes; in the liver, insulin inhibits gluconeogenesis by inhibiting key enzyme activities, while in the skeletal muscle it predominantly induces glucose uptake by stimulating glucose transporter 4 (GLUT4) translocation to the plasma membrane. In insulin resistance, FFA concentrations and the increase in fat accumulation significantly attenuate insulin-mediated glucose uptake in the skeletal muscle and glucose production within the liver. Together with impaired insulin secretion
and the resistance of peripheral tissue to the affects of insulin leads to type II diabetes mellitus.

The insulin signaling cascade is quite complex and upon insulin binding to the α subunit of its specific receptor, which belongs to a member of receptor tyrosine kinases, the inhibition of tyrosine autophosphorylation by the β subunit is released. The activated insulin receptor directly phosphorylates IRS-1 to -4 on specific tyrosine residues with IRS-1 and IRS-2 being the most important and crucial for glucose transport. The tyrosine phosphorylated IRS proteins then act as specific binding sites for molecules that contain the Src-2 homology-2 (SH-2) domain such as pyruvate dehydrogenase lipoamide kinase isoenzyme 1 (PDK1), SH2 domain containing protein tyrosine phosphatase (SHP2) and growth factor receptor bound protein-2 (GRB-2)/mammalian Son of Sevenless homolog (mSOS), a guanine nucleotide exchange factor. Upon binding to the tyrosine phosphorylated-IRS, they form signaling complexes to mediate further downstream signaling of which the PI3K-Akt cascade is mostly responsible for the action of insulin, glucose uptake, and inhibition of gluconeogenesis. PI3K is composed of a p85 regulatory subunit which binds to IRS proteins and a catalytic subunit, p110. This allows for phosphorylation of its substrate phosphatidylinositol(4,5)P$_2$ (PIP$_2$) on the 3’ position of the inositol ring to generate phosphatidylinositol(3,4,5)P$_3$ (PIP$_3$). Then the secondary messenger, PIP$_3$, recruits serine kinases PDK1, Akt and PKC to the plasma membrane via their PH
domain and upon their activation results in GLUT4 translocation to the membrane, glycogen synthesis via GSK3 phosphorylation, and lipogenesis by upregulation of the fatty synthase gene.\textsuperscript{136} In addition to activating the PI3K-Akt pathway, insulin can also activate the MAPK ERK as means to regulate gene expression, control cell growth and cell differentiation.\textsuperscript{135} In contrast to activating tyrosine phosphorylation of IRS, the serine phosphorylation on critical serine sites will \textit{inhibit} insulin signaling. Specific serine kinases that can phosphorylate IRS-1 include inhibitor of nuclear factor κ-B kinase β (IKK-β), JNK-1, and other members of the MAPK.\textsuperscript{139,140} Insulin is capable of activating JNK and other serine kinases and this is seen as a possible negative feedback mechanism for the signaling cascade. At the molecular level, there are many other mediators especially within the inflammatory system which may play a role in the inhibitory cross-talk for insulin signaling. These include suppressor of cytokine signaling (SOCS) 1 and 3, which during inflammation, are induced by interleukin (IL)-6 and lead to ubiquitinylation and degradation of the IRS proteins.\textsuperscript{141,142}

The pathophysiological effects of insulin resistance are multifaceted and complex and can vary from tissue to tissue. Notably, once compensatory hyperinsulinemia is established to maintain near normal glucose levels, there is increased sodium retention and over-activation of the sympathetic nervous system.\textsuperscript{143} This phenomenon helps contribute to the increased prevalence of hypertension in this patient population. Furthermore, insulin signaling plays a role in both NOS and ET-1 expression as shown in isolated endothelial cells from mice lacking vascular endothelial insulin receptors.
While the exact relationship remains unclear, it is evident from the literature that insulin resistance plays a role in diabetic-induced endothelial dysfunction and remains an important predictor of CHD and stroke.

**i. Hypertriglyceridemia**

In 1967, it was first demonstrated that there is a strong association between insulin resistance, the compensatory hyperinsulinemia and elevated fasting plasma triglyceride levels. Further research led to the discovery of the potential mechanism behind this relationship. Hyperinsulinemia was shown to trigger hepatic very low-density lipoprotein (VLDL)-triglyceride secretion, which in turn increases plasma triglyceride levels. It is postulated that in the insulin-resistant state, the liver is resistant to the effects of insulin with regard to lipoprotein metabolism and accordingly cannot inhibit VLDL-triglyceride secretion. In fact, acute infusion of insulin has been shown to directly suppress hepatic VLDL-triglyceride secretion in addition to causing a substantial decrease in adipose tissue lipolysis.

**G. Obesity and Inflammation**

According to WHO, obesity and metabolic syndrome is the fifth leading cause of death worldwide and as of 2008, there were approximately 1.4 billion overweight adults globally of whom at least 200 million are clinically obese. Diabetes and obesity have
been considered at least in part to be inflammatory conditions, with adipose tissue being the first organ to be affected.\textsuperscript{149} The primary etiology for obesity-induced inflammation is not completely understood, however one potential basis for the initiation of inflammation in obese individuals may be ER stress. Excess nutritional intake and obesity lead to ER stress within the liver and adipose tissue secondary to excess lipid accumulation and dysregulated energy metabolism.\textsuperscript{150} The ER stress activates the “stress response signaling network” known as unfolded protein response (UPR), which activates protective pathways such as apoptosis and inflammatory cascades and is an adaptive response of cells to a large metabolic load.\textsuperscript{151} The UPR occurs through three different transmembrane molecules which transmit the ER stress signal to the nucleus through a number of cytoplasmic signaling molecules and kinases with the downstream activation and transcription of pro-inflammatory genes.\textsuperscript{152} During ER stress, the protein kinase/endoribonuclease inositol requiring protein-1 (IRE1), a key transmembrane protein, initiates non-spliceosomal splicing of the mRNA transcription factor X-box binding protein-1 (XBP-1) that controls protective mechanisms to ER stress. Accordingly, IRE1 also can induce the inflammatory cascade by activating IKK-β, two different MAPKs—p38 and JNK, and the key inflammatory transcription factor, nuclear factor kappa B (NF-κB). The second molecule involved is the UPR is the membrane localized transcription factor, activating transcription factor-6, that is cleaved in response to ER stress and once cleaved, enters the nucleus to activate transcription of chaperone genes and further augment NF-κB transcription.\textsuperscript{153} The final molecule is PKR-like
eukaryotic initiation factor 2α kinase, which inhibits protein translation by phosphorylating transcription initiation factor eIF2α. This activates the alternative translation of activating transcription factor-4, which induces the production of inflammatory cytokines, stimulates NF-κB transcription, and inhibits translation of the NF-κB inhibitor, I kappa B alpha (IκBα). The ultimate goal of UPR is to re-establish ER homeostasis and facilitate proper protein folding with the assistance of chaperone proteins, arrest further protein synthesis and degrade mis-folded proteins. If the ER function does not recover, UPR will stimulate apoptosis signaling pathways for programmed cell death. Obesity-induced ER stress occurs secondary to metabolic flux, increased lipid stores, lipogenesis, increased insulin production from β pancreatic cells, and gluconeogenesis and all have a negative impact on cells causing ER stress. In β pancreatic cells, the excessive demand and ER stress for the cells to produce insulin in the insulin resistant state, ultimately leads to the decrease in insulin synthesis and β-cell apoptosis. In addition it leads to IRS serine phosphorylation and inhibition of insulin signaling. The ER stress hypothesis is supported by experiments using XBP-1+/− mice in which the protective effects towards ER stress are significantly attenuated and these mice are especially prone to insulin resistance. Moreover, use of chaperones to further reduce ER stress restores insulin sensitivity in obese mice. During in vitro experiments, ER stress was shown to downregulate expression of GLUT4 in cultured adipocytes and these data support the notion that ER stress affects insulin signaling,
likely through activating inflammatory signaling pathways in target cells, particularly adipocytes, which contributes to insulin resistance.

Inflammatory cytokines, chemokines and adhesion molecules have recently gained significant attention as potential therapeutic targets.\textsuperscript{156} Likewise, in obese individuals it is not only inflammation, but also oxidative stress within the adipose tissue that leads to insulin resistance.\textsuperscript{157,158} In addition, inflammation plays a central role in vascular disease, from plaque inception to progression and destabilization, which represents a significant paradigm shift from the old idea that vascular disease and atherosclerosis is a problem of lipid accumulation and oxidized cholesterol. The association of inflammation with obesity and insulin resistance dates back to 1993 when Hotamisligil \textit{et al} found that adipocyte expression of the pro-inflammatory cytokine tumor necrosis factor (TNF)-α was significantly increased in \textit{db/db} mice.\textsuperscript{159} TNF-α also effects insulin signaling through phosphorylation of S6K1 (p70S6K), which impairs the normal insulin response through serine phosphorylation of IRS-1 and inhibition of tyrosine kinase activity of the insulin receptor in adipocytes and hepatocytes.\textsuperscript{160} Another explanation that has recently emerged is that mice lacking a key phosphatase, phospho-tyrosine protein phosphatase 1B (PTP1B), were protected from TNF-α induced insulin resistance. This tyrosine phosphatase has been shown to tyrosine dephosphorylate and deactivate IRS-1 to balance the actions of the kinases involved in insulin signaling. Adipose tissue inflammation via high-fat diet and TNF-α, induced expression of PTP1B in adipose tissue, muscle, liver and hypothalamic arcuate nucleus
occurs through a mechanism involving NF-κB.\textsuperscript{161} Interestingly, sirtuin1 (SIRT1) was shown to inhibit PTP1B through repression at the chromatin level and improves insulin sensitivity whereas direct inhibition with a PTP1B inhibitor also improved insulin resistance and obesity-related complications\textsuperscript{162,163} (figure 5).

Recently, more and more evidence has emerged that obesity is associated with inflammation that is causally involved in the development of insulin resistance and diabetes. Obese patients have increased levels of hs-CRP, TNF-α, IL-6, monocyte chemotactic protein (MCP) -1 and IL-8 and leptin.\textsuperscript{164} Genetically altered mice lacking TNF-α, or the TNF-α receptor 1 gene were shown to be protected from insulin resistance both in diet induced obesity and a genetic obesity (ob/ob) model.\textsuperscript{165} Experiments using cultured murine adipocytes confirmed that TNF-α stimulates IKK-β and SOCS3, both of which negatively affect the insulin signaling cascade. Likewise, the obesity-induced IL-1β, also plays an important negative role in the signaling pathway.
Figure 7. Obesity-induced inflammation and disruption of the insulin-signaling cascade.

Inflammation induced by obesity leads to downstream disruption of the insulin signaling pathway. Excess nutrition leads to elevated lipids, free-fatty acids (FFA), long-chain fatty acids (LCFA) and endoplasmic reticulum (ER) stress. This causes activation of (protein kinase C [PKC]-θ, c-Jun-N kinase [JNK], inhibitor of κ B α [IkBα]), which activate inflammation in adipose tissue. Likewise, obesity itself is an inflammatory disorder, which increases levels of tumor necrosis factor-α, interleukin (IL)-1β, IL-6. This leads to decreased phosphorylated Akt and increase in serine phosphorylation of the insulin receptor substrate-1 (IRS-1), which inhibits effective insulin signaling.
One study showed that obese individuals with detectable levels of IL-1β and elevated IL-6 concentration had an independent risk (3.3-fold increase) of developing type II diabetes as compared to those with only elevated IL-6 levels.\textsuperscript{166} In fact, treatment with an IL-1β antagonist improved blood glucose levels, pancreatic β cell function, and inflammatory markers IL-6 and TNF-α.\textsuperscript{167} Using 3T3-L1 and human adipocytes, chronic treatment with IL-1β, induced insulin resistance through IRS-1 downregulation and decreased IRS-1 tyrosine phosphorylation.\textsuperscript{168}

The signal transduction induced by endothelial derived NO involves the downstream activation of sGC and cGMP within the VSMC, which activates its effector, the cGMP-dependent kinase. This kinase can then phosphorylate vasodilatory stimulated phosphoprotein (VASP) at serine\textsuperscript{239}. It has been recently shown that disruption of VASP phosphorylation or eNOS signaling in this pathway leads to adipose tissue inflammation, which can be attenuated by use of PDE-5 inhibitors and that this leads to an attenuated inflammatory response.\textsuperscript{169}

H. **Oxidative Stress and Reactive Oxygen Species**

Oxidative stress is a major risk factor in the onset and progression of insulin resistance to diabetes mellitus. Similarly, ROS and the cellular redox state have been increasingly shown to play a significant role in affecting a variety of biological signaling
pathways.\textsuperscript{170} ROS are formed by the reduction of molecular oxygen or oxidation of water to yield superoxide ($O_2^\cdot$) anion, hydrogen peroxide ($H_2O_2$) and hydroxyl radical (\cdotOH). Within various biological tissues, the primary source of ROS is derived from the mitochondria and NADPH oxidase (NOX).\textsuperscript{170} In some situations, moderate amounts of ROS are crucial for normal physiological processes, however in significant quantities they are capable of cellular damage to DNA, proteins, membranes and lipids. Despite many beneficial cardioprotective effects, NO can be potentially detrimental when contributing to the formation of reactive nitrogen species (RNS).\textsuperscript{170} The NO radical (NO\textsuperscript{\cdot}) is formed from NOS and can react with $O_2^\cdot$ to form peroxynitrite (ONOO\textsuperscript{\cdot}) which is an extremely potent oxidizing agent and is capable of severe cellular damage and oxidative stress. When the cellular capacity and efficiency to scavenge and remove ROS or RNS is reduced and/or there is a gross overproduction, the end result is oxidative stress.

The main redox buffer within the cell is glutathione and using the Nerst equation allows the intracellular redox potential to be determined. Increases in ROS leads to an imbalance of the cellular oxidation state thus altering the redox balance.\textsuperscript{171} Several studies have shown that the diabetic has extremely high levels of ROS and this abnormal state leads to the long term complications and progression associated with the disease.\textsuperscript{172} Both hyperglycemia and increased FFA intake are associated with
oxidative stress and activation of stress pathways such as NF-κB, JNK and p38 MAPK. This leads to an alteration of insulin signaling and potentiation of the inflammatory cascade (figure 8).

There are numerous studies which support that hyperglycemia in diabetic patients causes an increase in ROS production and a concomitant decrease in antioxidant defenses. Likewise, as a marker of oxidative stress it can cause membrane lipid peroxidation. In one study, investigators showed that blood glucose concentrations directly correlated with the degree of erythrocyte lipid peroxidation. Moreover, the same group went on to show that control of the plasma glucose levels in streptozotocin-diabetes induced rats lead to a decrease in membrane lipid peroxidation. The key sources of ROS generation within the vasculature are the electron transport chain (ETC), NOX, and eNOS uncoupling. In 2001, Brownlee and colleagues developed the “unifying hypothesis” of which the major premise is that in the diabetic, ROS produced within the mitochondria initiates the development of nearly all diabetic complications.
Figure 8. The effects of oxidative stress on insulin resistance.

In obesity and conditions of hyperglycemia, there is a significant increase in oxidative stress. This leads to activation of serine/threonine (Ser/Thr) kinases, which phosphorylates the insulin receptor and prevents effective insulin signaling. Similarly, the mitochondria produce ROS, which leads to an increase in uncoupling protein-2 (UCP-2). Mitochondrial dysfunction ensues which decreases ATP and fatty acid oxidation. Diacylglycerol (DAG) production increases, which can also activate the Ser/Thr kinases blocking insulin signaling at the IRS.
It further states that ROS generated within the mitochondria migrate to the nucleus, cause DNA damage that results in activation of poly (ADP-ribose) polymerase-1 (PARP-1), and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) which after undergoing ADP-ribosylation by PARP-1, is inactivated. The activation of PARP-1 by DNA damage promotes NADPH depletion and further ADP-ribosylation of other proteins. The polyol pathway is a mechanism in which glucose is reduced to sorbitol in an NADPH-dependent manner by the enzyme aldose reductase. Sorbitol is then oxidized to fructose by sorbitol dehydrogenase along with the reduction of NAD$^+$ to NADH. Under normoglycemia, aldose reductase has a low affinity for glucose but under elevated glucose conditions, it has a higher than normal production of sorbitol and therefore an overall decrease in NADPH.

The subsequent decline in GAPDH activity leads to a rise in glycolytic intermediates and is the primary means of hyperglycemic-induced injury as these intermediates activate various pathways and mechanisms including: the polyol pathway flux, hexosamine pathway, activation of the PKC cascade, intracellular formation of AGEs, and through an overproduction of ROS via the mitochondrial ETC (figure 9).
Brownlee’s unifying hypothesis of ROS-induced diabetic complications hinges around increased mitochondrial ROS production, which migrates to the nucleus to cause DNA damage. This causes PARP-1 activation and ADP-ribosylation of GAPDH and leads to an increase in glycolytic intermediates. This shuttles the intermediates into a number of pathways leading to hyperglycemic-induced injury.

The second mechanism by which hyperglycemia increases the oxidative milieu is through increased AGE formation. AGEs are formed by creating a Schiff’s base—through the binding of aldehyde or ketone groups of reducing sugars to free amino
groups of proteins. The Schiff’s base then forms a more stable ketoamine in the form of an Amadori product, which undergoes autoxidation to form reactive dicarbonyl intermediates such as 3-deoxyglucose, glyoxal and methylglyoxyl. These intermediates undergo further chemical rearrangements until the final irreversible AGE is formed. The AGE is noted to signal through the receptor for AGE (RAGE). One of the primary consequences of ligand-RAGE binding is activation of the NOX system and generation of ROS which then activates the pleiotropic transcription factor NF-κB causing pathological changes in gene expression. AGEs also play a significant role in failure of adequate angiogenesis as hyperglycemic conditions induces a decrease in transactivation by the transcription factor HIF-1α which is responsible for mediating the hypoxia stimulated increase in VEGF and eNOS within endothelial precursor cells within the bone marrow. Likewise, AGE binding to its receptor within endothelial cells was shown to alter expression of several genes including thrombomodulin, tissue factor and vascular cell adhesion molecule (VCAM)-1 thus increasing adhesion of inflammatory mediators to endothelial cells and inducing a pro-coagulant state.

Protein kinase C has various isoforms and is capable of phosphorylating a number of target tissues. Its function is largely dependent on Ca\textsuperscript{2+} ions and phosphotidylserine and its function can be enhanced by diacylglycerol (DAG). During hyperglycemia, DAG synthesis is significantly enhanced from glucose via triose phosphate secondary to the inhibition of the glycolytic enzyme GAPDH. The resulting
increase in PKC activity (β and γ isoforms) activates p38α MAPK and subsequently the protein tyrosine phosphatase, SH-2. In addition, PKC signaling can induce oxidative stress through decreasing NO bioavailability and altering the prostanoid profile characterized by an increase in TXA₂ and a reduction in PGI₂. Further activation of NOX by TXA₂ can uncouple eNOS leading to increases in intracellular ROS production. Likewise, elevated glucose can activate PKC inducing VEGF, TGF-β₁, fibronectin and type IV collagen, which has been implicated with overexpression of plasminogen activator inhibitor-1 (PAI-1), a fibrinolytic inhibitor.

Fatty acid oxidation is another pathway that contributes to oxidative stress and vascular complications in hyperglycemia and insulin-resistance. Known as the hexosamine pathway flux, an influx of fructose into this cascade whereby fructose-6-phosphate is diverted from glycolysis to provide the necessary substrate for the rate-limiting step, glutamine:fructose-6-phosphate amidotransferase (GFAT). GFAT then converts fructose-6-phosphate into glucosamine-6-phosphate, which can be converted into uridine diphosphate (UDP)-N-acetylglucosamine. Then specific O-linked acetylglucosamine transferases use O-linked acetylglucosamine to modify, in a post-translational manner, serine/threonine residues on various proteins within the cell. The resultant increase flux through the hexosamine pathway leads to upregulation of key genes such as TGF-α, TGF-β₁, and PAI-1. Additionally, this pathway may play a role in cardiomyocyte dysfunction and the development of cardiomyopathy as
increases in nuclear O-linked acetylglucosamine reduces sarcoplasmic reticulum Ca$^{2+}$ ATPase 2a mRNA levels and protein expression and could impair β-agonist induced inotropy.\textsuperscript{186}

The majority of ROS within the physiological system is produced by the mitochondrial ETC. In this process, electrons from NADH and FADH$_2$ produced during glucose and fatty acid synthesis are used to create an electrochemical gradient sufficient for the production of ATP.\textsuperscript{187} The rate of oxidative phosphorylation is dictated by energy demands of the cell and as cellular demands increase, production of NADH and FADH$_2$ must also increase for adequate ATP production. While normal function of the ETC is to produce ATP, it also serves as a source for O$_2^-$ formation.\textsuperscript{187} The primary sources for “electron leak” to oxygen occurs at complex I (NADH-CoQ reductase) and complex III (CoQH$_2$-cytochrome c reductase) whereas in the heart and lung tissues, complex III appears to be the primary source of O$_2^-$ formation.\textsuperscript{181}

The mitochondria however are equipped with an antioxidant defense mechanism to protect against the lethal effects of ROS. Within the intermembrane space is the cytosolic superoxide dismutase 1 (SOD1) also known as Zn,Cu-SOD and within the mitochondrial matrix is superoxide dismutase 2 (SOD2) also known as Mn-SOD, and both are capable of rapidly catalyzing the formation of O$_2^-$ to hydrogen peroxide
Hydrogen peroxide can readily diffuse across membranes and can be further degraded by antioxidants such as catalase, peroxiredoxins (Prx1, Prx2) and glutathione peroxidases (Gpx1-4). Several studies have recently shown that mitochondrial dysfunction in genetic diseases correlates with a propensity to develop insulin resistance. Similarly, the oxidative capacity of a cell correlates with the number and density of mitochondria and relates to the reduction in expression of mitochondrial proteins involved in mitochondrial biogenesis and ATP production. A reduction in fatty acid oxidation by the mitochondria caused by either a decrease in mitochondrial biogenesis or dysfunction, leads to increased acyl-CoA and DAG. This in turn activates stress-induced serine/threonine kinase activity which inhibit glucose transport. According to Brownlee's unifying hypothesis, hyperglycemia mediated increases in reducing equivalents NADH and FADH$_2$ enhances flux through the ETC and increases the mitochondrial membrane potential (Δψm) which partially inhibits flux through complex III. This allows diversion of electrons from a reduced Coenzyme Q to O$_2$ thus forming O$_2$·⁻ anion. Rosca et al recently published data supporting this hypothesis in that hyperglycemia led to a ROS-dependent partial inactivation of complex III and downregulation of genes encoding for complex III. Likewise, oxidative stress can cause an increase in uncoupling protein-2 (UCP-2), a protein that upon activation, allows for protons to leak across the mitochondrial membrane hence contributing to the
dissipation of the $\Delta \psi_{m}$.\textsuperscript{191} A number of animal and \textit{in vitro} models have shown that UCP-2 is a negative regulator of glucose-stimulated insulin secretion.\textsuperscript{192} In a transgenic diabetic model, UCP-2$^{-/-}$ mice had improved pancreatic $\beta$-cell function with improved insulin secretion despite being on a high fat diet.\textsuperscript{193} Likewise, \textit{in vitro} models have confirmed these findings as cells subjected to hyperglycemic conditions demonstrated significant elevations of UCP-2 expression.\textsuperscript{194} The elevated risk of developing CAD in obesity, insulin resistance is caused, at least in part, by mitochondrial ROS production from FFA and antioxidant inactivation by ROS.

Endothelial nitric oxide synthase is an enzyme made up of two separate domains, a reductase domain which contain co-factors FAD, FMN, and NADPH and an oxidase domain containing a heme-active site.\textsuperscript{195} It is a calcium-dependent flavoprotein which catalyzes the oxygen dependent oxidation of L-arginine to form NO. In normal conditions, NADPH oxidation generates an electron, which is transferred to FAD and then FMN and finally the oxidase domain for NO production.\textsuperscript{195} In eNOS uncoupling, NADPH oxidation becomes “uncoupled” from the oxidation of L-arginine and electrons flow directly from the flavoproteins to molecular oxygen to form $O_2^-$. The most common cause for is low levels of the cofactor BH$_4$, as it serves to stabilize the dimeric structure of the enzyme and facilitates formation and stabilization of the Fe$^{2+}$-O$_2$ intermediate.\textsuperscript{196} During hyperglycemia, higher than normal levels of ONOO$^-$ oxidizes BH$_4$ to BH$_2$. Likewise, eNOS uncoupling plays a key role in reducing vascular tone and leading to
the development of micro- and macro-vascular complications associated with diabetes.\textsuperscript{197}

Similar to NOX, xanthine oxidoreductase is another key enzyme capable of forming oxidative stress. It is a ubiquitous enzyme and exists in two different forms—xanthine dehydrogenase, which catalyzes the oxidation of hypoxanthine to xanthine using \( \text{NAD}^+ \) as an electron acceptor, and xanthine oxidase, which is formed via oxidation of sulfhydryl residues or proteolytic cleavage of the parent enzyme.\textsuperscript{195} Xanthine oxidase serves as an important source of oxidative stress during I/R injury and can also catalyze the oxidation of hypoxanthine to xanthine.\textsuperscript{198} In a study on diabetic animals, higher than normal levels of xanthine oxidase were found in the systemic circulation and ROS levels were significantly attenuated in aortic rings after treatment with the xanthine oxidase inhibitor, allopurinol.\textsuperscript{198,199}

In summary, ROS are involved in a number of pathological processes and the “unifying hypothesis of diabetes” provides an explanation and a link between ROS, mitochondrial dysfunction, oxidative stress and diabetic complications. Hyperglycemia-induced glycolytic flux, which is inhibited, subsequently has its intermediates diverted to pathological pathways such as the PKC cascade, hexosamine pathway, AGE formation and polyol pathway all of which serve as critical mechanisms behind ROS and oxidative stress-induced vascular injury. Likewise, mitochondrial \( \text{O}_2^- \) generation leads to cell
death, and PKC-\(\gamma\) activation by NOX upregulation—one of the critical mechanisms behind diabetic oxidative-stress induced complications.

I. **MicroRNAs**

MicroRNAs (miRs) are a large class of phylogenetically conserved, non-coding single-stranded RNA molecules of 19-25 nucleotides that negatively regulate gene expression by base pairing with the 3’ untranslated region (UTR) of messenger RNA (mRNA), and inducing mRNA degradation or translational inhibition.\(^\text{200}\) Once considered simple by-products of mRNA transcription, the first animal miR was described in 1993 as a regulator of developmental timing in *Caenorhabditis elegans*.\(^\text{201}\) MiRs that bind to mRNA targets imperfectly regulate their target gene at the protein translation level whereas perfect miR-to-mRNA binding induces target mRNA cleavage. Based on new computational algorithms, approximately 60% of human transcripts are potentially regulated by miRs as they contain miR binding sites within their 3’ UTR.\(^\text{202}\) MiRs are transcribed as precursor mRNAs from intergenic, intronic or polycistronic genomic loci by either RNA polymerase II or RNA polymerase III. This forms the primary miRNA, also known as pri-miRNA, and subsequently forms a stem-loop structure that is cleaved and further processed by the enzyme Drosha and its cofactor Pasha.\(^\text{202}\) In the non-canonical pathway, miRs are generated directly through splicing of introns to form hairpin structures upon refolding. These pre-miRs are then transported out of the nucleus by
exportin 5 and a RAN-GTP-dependent process to the cytosol where they are processed further by Dicer and transactivation-response RNA binding protein RNAse III enzyme complex to form mature double-stranded 22-25 base pair miRNA. Argonaute proteins then facilitate the unwinding of the miR duplex to allow incorporation of the miR-targeting strand into the argonaute-containing RNA-induced silencing complex (RISC). This subsequent complex, RISC-miR assembly, can then be guided to its target sequence in mRNA to become a “post-transcriptional regulator.” The recognition of the target is primarily driven by the Watson-Crick base-pairing of nucleotides 2-8 in the mature miR which is termed the seed sequence, to the target sequence within the 3’ UTR of the mRNA. Further base-pairing leads to additional affinity and efficiency. A single miR can potentially bind to more than 100 target mRNAs and multiple miRs can cooperate to fine tune the expression of a single transcript. Although this area of research has only just begun, the deregulation of miR biogenesis and function has already shown to play a critical role in a variety of physiological and pathophysiological processes especially in cardiovascular diseases and the metabolic syndromes such as insulin resistance, lipid dysmetabolism and obesity.203

i. **MicroRNAs in Cardiovascular Diseases**

Microarray studies have identified specific miRs that are upregulated and/or downregulated in specific pathological conditions such as vascular inflammation and
include: miR-126, miR-17, miR-92a and miR-155. In a recent study, each of these inflammation-associated miRs were significantly downregulated in patients with IHD when compared to healthy controls, whereas the cardiac-muscle enriched miR-133a and miR-208a were both significantly higher in patients with CHD.203

One of the first miRs identified to have a role in I/R was miR-1 because it is preferentially expressed in adult cardiomyocytes and skeletal muscle. MicroRNA-1 is involved in cardiac development and heart disease and regulates a number of key functions.200 These include: apoptosis through targeting synthesis of HSP60, HSP70 and Bcl-2, and arrhythmogenesis by targeting the KCNJ2 and GJA1 genes.204 The KCNJ2 gene encodes kir2.1, a subunit of the potassium ion channel, whereas the GJA1 gene encodes connexin43, a major component of the gap junction. Moreover, decreased expression of kir2.1 and connexin-43 delays membrane repolarization and conduction, thereby increasing the risk of fatal arrhythmias.205-208 In two different rat models, miR-1 was found to be significantly upregulated in response to reperfusion injury and acute MI.207,209 Likewise, oxidative stress and subsequent miR-1 upregulation decreases cardioprotective HSPs including: HSP60 and HSP70. In H9c2 cells, overexpression of miR-1 augmented H$_2$O$_2$-induced apoptosis whereas inhibition of miR-1 conferred resistance of cells to H$_2$O$_2$.206

MicroRNA-126 may play a critical role in AMI. The survival rate after in vivo I/R injury in miR-126$^{-/-}$ mice was significantly reduced as compared to the wild-type.210 Our
lab has previously evaluated the induction of miRs in response to IPC. We induced IPC with short bursts of global ischemia (2 bursts of 30 sec ischemia followed by 90 sec reperfusion) in a Langendorff isolated perfused heart model and found a significant induction miR-1, miR-21 and miR-24.\textsuperscript{211} To determine a causal relationship between IPC-induced endogenous miRs and cardioprotection, pools of extracted miRs were taken from non-preconditioned and preconditioned hearts and directly injected \textit{in vivo} into the risk zone of the LV wall in mice. Forty-eight hours later, these mice were subjected to \textit{in vivo} I/R injury. We demonstrated that the IPC-derived miR-injected group had a cardioprotective phenotype with significantly smaller infarcts compared to saline treated or non-IPC-derived miR injected group (18.8±2.5 vs. 37.5±2.2 vs. 39.3±2.3%, p<0.05; n=6). It is also noteworthy that there was a significant upregulation of eNOS protein (92±8.1%), HSP transcription factor-1 (42.7±3.0%) and HSP70 (102.3±8.9%), 48 h after treatment with miR derived from IPC hearts.

In a study by Dong \textit{et al}, it was shown that miR-21 is upregulated in the risk-area after I/R injury and is downregulated within infarcted myocardium.\textsuperscript{202} They found that IPC abrogated the downregulation of miR-21 in the infarcted area whereas miR-21 overexpression leads to reduced apoptosis, smaller infarct sizes, and improved LV remodeling at 2 weeks. The mechanism of miR-21 cardioprotection has recently been elucidated and found to be downstream of the PI3K-Akt signaling cascade. Sayed \textit{et al} have demonstrated that miR-21 is a downstream effector of Akt which upon phosphorylation, mitigates apoptosis.\textsuperscript{212} Transgenic mice lacking miR-21, showed Akt
mediated suppression of Fas ligand (a key activator in the apoptosis cascade) and abrogated caspase-8 activity. This provided direct evidence that Akt-mediated antiapoptotic effect on Fasl and caspase-8 may be directly mediated through miR-21.

In a study by Zhang et al, showed that GATA-4, a key transcription factor in the heart, could activate miR-144/451. Using a cardiomyocyte model of SI/RO, they demonstrated that individual expression of miR-144 or miR-451 augmented cell survival, which then could be further improved upon by overexpression of either miR. Congruent with these findings, knock-out of either miR revealed attenuated cell survival. Conversely, miR-320 has been shown to be downregulated in mouse hearts after I/R Injury and overexpression enhances cell death and apoptosis in isolated cultured rat cardiomyocytes subjected to SI/RO. Moreover, genetically engineered mice with cardiac-specific overexpression of miR-320 have increased infarct size and apoptosis after I/R injury whereas knockdown of miR-320 with use of an antagonim led to a significant reduction in both infarct size and apoptosis. It is proposed that miR-320 exerts its effect by downregulating HSP20, a protein known to protect the myocardium against I/R injury, thus indicating HSP20 as a possible putative target of miR-320. In a recent study by Wang et al, miR-499 was shown to inhibit apoptosis and infarct size induced by anoxia and coronary ischemia through mechanisms involving the apoptotic pathway including p53, cacineurin, and Drp1. They went on to show that an antagonim of miR-499 could produce the opposite effect and increase apoptosis and infarct size. Hullinger et al showed that miR-15 is significantly upregulated 24 h after
ischemic injury in the infarcted region and increased cardiomyocyte apoptosis.\textsuperscript{217} Using locked nucleic acid (LNA)-modified oligonucleotide modified anti-miR-15, inhibition of miR-15 caused a dose-dependent cardioprotective effect in both murine and porcine models of I/R injury.

In the recently published prospective study by Zampetaki \textit{et al}, baseline levels of 19 candidate miRs were identified in 820 participants in the Bruneck population and over a 10 year period (1995-2005), they sought to explore if any association existed between circulating miRs and incident MI.\textsuperscript{218} In a multivariable Cox regression analysis, miR-126 showed a strong positive correlation with MI hazard ratio (HR) of 2.69 [(95\% confidence interval (CI) of 1.45 to 5.01, \textit{p}=0.002] and miR-223 and miR-197 had a strong negative association with MI \textit{[HR} 0.47, 95\% CI 0.29 to 0.75, \textit{p}=0.002], and [HR 0.56, 95\% CI 0.32 to 0.96, \textit{p}=0.036]. They further suggested upon further I/R analysis in healthy volunteers that these 3 miRs are all part of the same miR pool and that endothelial enriched miR-126 is part of a miR signature associated with the incidence of MI in the general population independent of previous cardiovascular disease, sex or cardiovascular risk factors.

The exact mechanism behind individual miRs and their effect on preconditioning and other signaling pathways need to be fully elucidated before specific therapies to modulate expression are taken further. However once these intricacies have been
delineated, targeted miR or antagonir delivery may have tremendous therapeutic potential in reducing long-term complications associated with CHD.

ii. MicroRNAs in Glucose Homeostasis and Insulin Resistance

Microarray studies have highlighted certain miRs that promote insulin resistance, regulate glucose metabolism and insulin sensitivity. Likewise, many have been directly implicated in the insulin signaling and glucose uptake pathways. For example, miR-375 is required for the maintenance of β- and α- pancreatic cells in mice and was found to decrease insulin exocytosis and secretion through repression of the myotrophin gene—a gene involved actin depolymerization and potentially vesicular fusion. Additionally it affects downstream signaling through inhibition of PDK1, an intermediary in the insulin signaling pathway. MicroRNA-126 has been shown to inhibit IRS-1 and accordingly promote insulin resistance as another mechanism of insulin regulation.

In a study by Lu et al, they found that in LV tissue samples taken from patients with and without type 2 diabetes and from patients with LV dysfunction, quantitative analyses of 155 miRs revealed that miR-223 was consistently upregulated in the diabetic hearts. They went on to evaluate the downstream physiological effects of miR-223 on glucose metabolism in a rat neonatal cardiomyocyte model using adenoviral-mediated overexpression of miR-223 and found that glucose uptake was significantly increased.
Next they looked at the effects on the insulin signaling pathway with miR-223 overexpression and found that neither PI3K, nor AMPK activity increased, however GLUT4 protein expression did increase. These findings were confirmed in vivo using siRNA knockdown of GLUT4 and a synthetic inhibitor of miR-223.

Several miRs are preferentially expressed within the pancreas but the most notable and abundantly expressed in the islet cells is miR-375. This miR, under the control of transcription factors pancreatic and duodenal homeobox-1 (Pdx-1), also known as insulin promoter factor-1, and neurogenic differentiation-1 (NeuroD1), plays a negative role in glucose-induced insulin secretion.\textsuperscript{221} It affects the final step in the secretory pathway and this is partly attributed to expression of myotrophin, a gene involved in insulin-granule fusion. Additionally, miR-375 has also been shown to contribute to glucose homeostasis in genetically modified animal models lacking miR-375 which became severely hyperglycemic, glucose intolerant yet in spite of normal insulin secretion and clearance.\textsuperscript{220, 221}

Evidence has also shown that prolonged hyperglycemia can lead to aberrant cellular signaling through the activation of pro-inflammatory pathways and fibrosis that eventually lead to long term cardiovascular morbidity and mortality.\textsuperscript{149, 224} Rat neonatal myocytes under conditions of hyperglycemia have elevated levels of miR-1 and miR-206 and through modulation of MEK1/2, negatively regulate HSP60 and contribute hyperglycemia-induced cardiomyocyte apoptosis.\textsuperscript{225} Other miRs upregulated under
hyperglycemic conditions include: miR-124a, miR-107 and miR-30d whereas miR-296, miR-484 and miR-690 are downregulated. Specifically, miR-30d overexpression causes a reduction in insulin gene expression, hence suggesting that perhaps this miR may play a role in regulating key transcription factors for insulin biosynthesis. He et al showed that the miR-29 family is upregulated in the adipose tissue as well as the skeletal muscle of diabetic Goto-Kakzaki rats and that this elevation could be recreated by incubating 3T3-L1 adipocytes with insulin and subjecting them to chronic hyperglycemia.226

Let-7 was one of the miRs discovered and it is implicated in both malignancy and pluripotency.227 In the mouse, there are 12 genes that encode the Let-7 family which include nine different miRs [Let-7a, Let-c, Let-7f—which are encoded by two genes; Let-7b, Let-7d, Let-7e, Let-7i, and miR-98—all encoded by one gene]. As all family members share a common seed sequence, they are believed to exert similar functions.227 In a recent study by Frost et al, transgenic mice were created with tissue-specific overexpression of Let-7. Mice with global overexpression of Let-7 had lower body weight and size with impaired glucose tolerance secondary to diminished glucose-induced insulin secretion.228 Using LNA-modified anti-miRs to inhibit Let-7 activity, they found improvements in blood glucose levels and insulin resistance in obese mice. In addition after anti-miR treatment, these obese mice were found to have an increase in lean body mass, increase in muscle mass, without an increase in fat mass or any ectopic fat deposition. They concluded that Let-7 may in fact regulate glucose
metabolism in a number of organs and in the future, *Let-7* anti-miR therapy may be a feasible treatment option for obese diabetic patients.\(^{228}\)

MicroRNA-103 and miR-107 are introns located in the panthotenate kinase 1 (PANK1), PANK2, and PANK3 genes. Bioinformatics analyses have shown that both of these miRs probably act synergistically with PANK in regulating Acetyl CoA and lipid metabolism. MiR-107 was first associated with glucose dysmetabolism and diabetes mellitus in 2003 by Tang *et al* when they showed that miR-107 was significantly upregulated in a β-pancreatic cell line under hyperglycemic conditions.\(^{229}\)

Recently, Trajkovski *et al* reported that Cav-1, which influences lipid raft signaling, is a regulator of the insulin receptor and is the key target of miR-103/107 as it was upregulated upon inactivation of both miRs using antagomir therapy. Moreover, this was concomitant with stabilization of the insulin receptor, enhancement of insulin signaling and improvement of insulin-stimulated glucose uptake.\(^{219}\) Using recombinant adenovirus to overexpress miR-103 and -107, they found a significant rise in random and fasting blood glucose levels and insulin levels. Similarly, glucose tolerance was decreased after *i.p.* glucose load and insulin sensitivity also decreased when compared to controls. In contrast, using antagonirs against miR-103 and -107, the found hepatic glucose production decreased and glucose uptake by adipose tissue increased. Hence, silencing of miR-103 and -107 through antagonirs enhanced hepatic insulin sensitivity and adipose tissue.\(^{219}\) Conversely, it is also possible the miR-103 and -107 only exert
some of their effects on insulin signaling and glucose homeostasis through negative regulation of Cav-1 as both miRs have recently been shown to also strongly inhibit the miR processing enzyme, Dicer. Some experiments using Dicer−/− mice have revealed that this enzyme is necessary for the development and maintenance of pancreatic cells and insulin signaling.

Caveolin-1 is the primary protein of caveolae, which are lipid and cholesterol enriched vascular invaginations of the plasma membrane. Peptides within the scaffolding domain of Cav-1 have been shown to enhance insulin receptor kinase activity, whereas Cav-1−/− mice develop insulin resistance on a high fat diet. Data from Trajkovski et al supports that Cav-1 is a direct target of miR-103 and they showed that silencing of miR-103 and -107 resulted in a significant increase in Cav-1 levels within adipose and hepatic tissue but not skeletal muscle. This was supported by increases in insulin-stimulated phosphorylated Akt and insulin receptor-β. They concluded that miR-103 and -107 negatively regulate insulin sensitivity and one possible mechanism may be through downregulation of Cav-1, which diminishes the number of insulin receptors in caveolae-enriched plasma membrane and prevents effective downstream insulin signaling.

In adipogenesis, miR-103 exhibits a nine-fold increase in expression in early 3T3-L1 adipocytes and increases lipid droplet formation when ectopically expressed whereas the exact role of miR-107 in adipogenesis is still unclear. However, both miR-
103 and miR-107 levels are significantly upregulated in adipogenesis, whereas they decrease in adipocytes taken from diet-induced obese animals. This was the first time miRs involved in adipogenesis were shown to have an inverse expression pattern in obesity.

### iii. MicroRNAs in Lipid Dysmetabolism

The maintenance of metabolic and energy homeostasis is critical for normal physiology and health; abnormalities and dysregulation have been shown to lead to obesity and insulin resistance. Likewise, defects in cholesterol and fatty acid synthesis and use are associated with the atherosclerotic process prevalent in type II diabetes mellitus. A number of recent novel studies have found that miRs play a pivotal regulatory role through post-translational repression of key energy homeostatic processes. Additionally, abnormal expression of miRs in response to intrinsic factors (genetic or epigenetic) or extrinsic factors (environmental cues or stress) may contribute to aberrant gene expression patterns and accordingly have been implicated in contributing to the development and progression of atherosclerotic disease, loss of endothelial integrity, and VSMC hyperplasia. Recently the direct regulation of insulin sensitivity and glucose homeostasis by miRs has been shown in obese, diabetic mice which have upregulated expression of miR-103 and miR-107. Moreover, miRs have
also been well characterized in their regulatory role in cholesterol and lipid homeostasis, especially the liver specific miRs, miR-122 and miR-33a and miR-33b with their host gene, sterol regulatory element binding protein (SREBP).

Lipids are the structural components of cell membranes and are critical for metabolism, energy storage, and even cellular signaling. Lipids such as cholesterol and FFAs are synthesized by the liver once taken up in the gastrointestinal tract and the regulation process is under strict control through feedback inhibition by end-products and transcription factors such as SREBPs.\textsuperscript{236} Cholesterol is transported within the plasma by various lipoproteins such as LDL, VLDL and high-density lipoprotein (HDL). Low-density lipoprotein transports cholesterol to the peripheral tissues where it can be taken up via the LDL receptor. HDL however removes cholesterol from the periphery and returns it to the liver for further metabolism in a process called reverse cholesterol transport (RCT). MicroRNA-122 was the first miR to be linked to hepatic and cholesterol metabolism. Two studies show that antisense targeting of miR-122 significantly (approximately 25-30\%) reduces circulating plasma cholesterol levels by altering gene expression of 3-hydroxy-methylglutaryl-CoA-reductase.\textsuperscript{237,238} Likewise, using antisense based silencing of miR-122, shows decreased hepatic cholesterol and fatty acid biosynthesis in addition to increasing fatty acid β-oxidation.\textsuperscript{237} This lead to lower total circulating cholesterol and triglyceride levels in mice on a high-fat, Western diet. Given these promising results, LNA technology was utilized to antagonize miR-122 in non-human primates and was found to be non-hepatotoxic and resulted in lower circulating
cholesterol. However, lower levels of LDL came at the expense of lower levels of the cardioprotective HDL.

One of the most extensively studied regulators of cholesterol and lipid metabolism is the SREBP family of basic helix-loop-helix leucine-zipper (LZ) transcription factors (SREBP-1a/c and SREBP-2). Together they regulate and influence the biosynthesis and trafficking of fatty acids, cholesterol, triacylglycerols and other lipid metabolites and co-factors such as acetyl-CoA. The SREBP-encoding genes were found to be host genes to highly conserved miRs that largely have overlapping target gene sets and only differ by two nucleotides—miR-33a (SREBP-2 gene) and miR-33b (SREBP-1 gene). Current data suggest that miR-33a/b regulate cholesterol and lipid homeostasis in cooperation with their host genes by targeting the ATP-binding cassette (ABC) A1 cholesterol efflux pump for translational repression/mRNA degradation, thus leading to increased levels of intracellular cholesterol. The ABCA1 pump is a key mediator for cholesterol efflux from the liver to apoprotein A-1 for the generation of HDL. Likewise, it plays a significant role in the peripheral tissue by mediating HDL trafficking back, also known as RCT, to the liver for bile acid synthesis. Recently, Najafi-Shoushtari et al showed that injection of LNA-antisense oligonucleotides against miR-33a significantly increased HDL levels in mice on a Western-type diet. Similarly, other groups have showed that lentivirus mediated overexpression of miR-33a significantly reduced circulating HDL, whereas genetic deletion of miR-33a increased HDL levels.
MircoRNAs are critical regulators of normal physiology and the development of pathophysiology and recently their function in the metabolic homeostasis has emerged to the forefront in lipid research. To date, miR-122 and miR-33 are the best-characterized miRNAs for the maintenance of normal cholesterol and lipid homeostasis. As this field continues to development, novel discoveries yield therapeutic concepts and strategies for the treatment of metabolic syndrome through targeted inhibition or overexpression of miRs, raising considerable excitement for both scientists and clinicians alike.
HYPOTHESIS AND SPECIFIC AIMS

Hypothesis # 1: Chronic treatment with the long-acting PDE-5 inhibitor TAD and the synthetic curcumin analogue, HO-3867, will improve fasting glucose levels and insulin tolerance in leptin receptor null mice through increased cGMP dependent kinase signaling and downregulation of key inflammatory cytokines. Accordingly, there will be a potent anti-inflammatory effect seen which will improve glycemic parameters, determined by fasting glucose levels, glucose and insulin tolerance tests. Likewise when the two compounds are combined, given each compound works by different pathways, the potential benefit may be greater than when each agent is given individually. In addition, given data supports that curcumin upregulates antioxidant genes such as Nrf-2 and HO-1 and that PDE-5 therapy improves NO bioavailability through cGMP-dependent signaling, these potent agents together may significantly attenuate cellular oxidant stress. The glycemic improvements that we have already shown with TAD leads to an increase in cellular signaling through the PI3K-Akt pathway and AMPK activation. Accordingly, we will assess for protein expression of activated/phosphorylated Akt and AMPK in TAD treated groups. These two key proteins play a key role in insulin signaling, energy metabolism and in cardioprotection.
Specific Aim #1: Chronic treatment with TAD and HO-3867 will reduce fasting blood glucose levels, improve insulin and glucose tolerance, attenuate inflammation, cell necrosis, oxidative stress, ROS formation and accordingly preserve the mitochondrial membrane potential ($\Delta \psi_m$) after I/R injury or SI/RO.

Rationale

The goal of this aim is to show that chronic treatment with TAD and HO-3867 will improve glucose and insulin tolerance, attenuate inflammation by restoring NO signalling, and enhance glucose utilization. We further plan to show that this combination treatment will reduce oxidative stress, ROS formation, inflammation and preserve the $\Delta \psi_m$ after 40 min SI and 1 h or 18 h of RO. Curcumin has been shown to be a free radical scavenger and potent antioxidant and inhibitor of oxidative DNA damage. In one study, curcumin inhibited oxygen free radical production caused by high glucose concentrations and increased glucose utilization by erythrocytes. Our preliminary results supported the findings that TAD therapy for 4 weeks can ameliorate pro-inflammatory cytokines and reduce fasting glucose levels.$^{244}$ We propose that TAD therapy will attenuate oxidative stress, ROS, and preserve the $\Delta \psi_m$ in addition to providing a potent anti-inflammatory effect and upon combining this with HO-3867 will be additive if not synergistic given the anti-oxidant and anti-inflammatory properties of curcumin. In our preliminary experiments, two groups ($n=6$/group) of leptin receptor null,
homozygous db/db mice (strain B6.Cg-m +/-Lepr<sup>db</sup>/J) were treated for 4 weeks and were assigned to either TAD (1mg/Kg) or 10% DMSO and similarly had weekly fasting blood glucose levels, body weight, and at the end of the treatment period were subjected to I/R in a Langendorff isolated perfused heart model. Blood samples were collected and tested for plasma levels of circulating inflammatory cytokines and triglyceride levels. As seen in figures 10, 12-13, TAD lead to a significant reduction in infarct size, fasting glucose levels, circulating levels of key inflammatory cytokines such as TNF-α, IL-1β, and IFN-γ. Likewise, chemokines MCP-1, MIP-1α and MIP-1β were also reduced. This hypothesis generating data led to the specific aim #1. The preliminary hypothesis generating results are given below and were recently published.²⁴⁴
The db/db mice treated with TAD showed a significant decrease in fasting plasma glucose levels (292±31.8 mg/dL vs. 511±19.3 mg/dL) compared to controls after the treatment period, n=6/group.
Body weight remained unchanged between control $db/db$ and TAD treated $db/db$ mice 41.4±1.2 g vs. 45.8±1.7 g, $n=6$/group.

Figure 11. Mean body weight after 4 weeks of TAD treatment.
Following I/R, infarct size was reduced after chronic treatment with TAD as compared to vehicle (DMSO)-treated db/db mice, 23.2±1.5% vs. 47.8±3.7%, p<0.01; n=6/group.
Figure 13. Reduction of cytokines and chemokines after TAD treatment.

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Key inflammatory cytokines and chemokines such as IL-1β, TNF-α, and chemokines, MIP-1β, and MCP-1 were attenuated with chronic TAD treatment and there was an increase in IL-10 levels when compared to the control group.
Figure 14. Rate-force product as % of pre-ischemia baseline.

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The post-ischemic rate-force product (expressed as % of pre-ischemic baseline compared to the control group) was not changed, 72.2±2.9% vs. 63.8±3.8%, p=0.10; n=6/group.
The coronary flow rate improved in the TAD treated db/db mice (147.4±3.2% of the pre-ischemic baseline vs. 98.3±1.2% in the DMSO treated mice, p=0.005; n=6/group. Baseline and post-ischemic coronary flow rates ranged from 1.8 to 2.1 mL/min and 1.7 to 1.9 mL/min in the TAD group, respectively and 1.7 to 2.0 mL/min and 1.5 to 1.6 mL/min in the control group, respectively.
After 18h of RO, apoptosis was also inhibited as indicated by reduced number of TUNEL-positive cells in TAD treated cardiomyocytes 23.4±1.9% vs. 8.8±1%, p<0.001.

Figure 16. Number of TUNEL positive (Apoptotic) cells after SI/RO.

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Figure 17. LDH assay to determine necrotic cells after SI/RO.

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An LDH assay was also performed to determine the amount of necrosis after SI/RO. Isolated cardiomyocytes from TAD treated mice showed much lower LDH release into the cell medium than mice treated with DMSO.
After 40 min of SI and 1 h of RO, the percent of trypan blue-positive (necrotic) cardiomyocytes was 59.8±1.5 in the control group. Treatment with TAD resulted in decrease of ~64% of trypan blue-positive cardiomyocytes, i.e., 21.3±1.5%; p<0.001 vs. control.

Figure 18. Number of Trypan Blue positive (necrotic) cells after SI/RO.

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TAD treated cardiomyocytes had a 44.3±2.5 % increase in PKG activity compared to C57BL/6 non-treated controls whereas the DMSO treated group had a 16.7±1.8% decrease compared to the non-diabetic control, p <0.001; n=4.
**Hypothesis #2:** Recent studies have shown that two related miRs, miR-103 and -107, negatively regulate insulin sensitivity and glucose metabolism and are upregulated in insulin resistance and obesity. In diabetic mice, silencing of both miRs improves insulin signaling and glucose homeostasis. Based on predicted miR targets, target downregulation scores, and observed expression patterns, it has been shown that miR-103/107 has a complimentary sequence alignment with caveolin-1 (Cav-1), which is a key protein making up caveolae within plasma membranes. Cav-1 has been shown to play a critical role in stabilizing the insulin receptor and enhancing insulin signaling. Hence, we contemplated that downregulation of Cav-1 by increased miR-103/107 expression in obese, diabetic mice may have a role in preventing effective insulin signaling and glucose homeostasis. In addition, it is known that PDE-5 inhibitors effect eNOS expression and that eNOS itself temporally and spatially regulates caveolar microdomains that facilitate signal transduction. We therefore contemplated that downregulation of Cav-1 by increased miR-103/107 expression in obese, diabetic mice may have in a role in preventing effective insulin signaling and glucose homeostasis and that chronic daily treatment with TAD may decrease the expression of miR-103/107. We further speculate that the mechanism for improving glucose regulation may be through increased expression of Cav-1 protein and that increased Cav-1 would lead to insulin receptor stabilization and improved insulin signaling.
**Figure 20. Complimentary sequence alignment between miR-103/107 and Cav-1.**

The above figure depicts the complimentary sequence alignment between both miR-103 and miR-107 and Caveolin-1 thus making it, based on target downregulation scores and observed expression patterns, the likely downstream target gene.

**Specific Aim #2:** To demonstrate that chronic treatment with TAD will decrease myocardial expression of miR-103/107, thus increasing mRNA and protein expression of its downstream target Cav-1. This may be one of the potential mechanisms by which TAD improves glucose homeostasis and insulin sensitivity in obese diabetic mice.
Rationale

MicroRNAs are novel endogenous short single-stranded non-protein encoding RNA’s with 19-25 nucleotides in length and been shown to play a key regulatory role in post-transcriptional gene expression. In recent years miRs are being increasingly recognized as potential therapeutic targets for a variety of diseases in addition to possible biological markers for making early diagnoses in a number of diseases. Given that miRs -103 and -107 have been shown to play a role in glucose metabolism and insulin sensitivity, and cGMP-dependent signaling is a key signaling pathway that upon activation can improve glucose homeostasis, we proposed that TAD therapy would decrease myocardial expression of miR-103 and -107 and since recent data supports these miRs target Cav-1, would increase mRNA and protein Cav-1 expression.
CHAPTER 3

METHODS

Thirty-two leptin receptor null, homozygous \( db/db \) mice (strain B6.Cg-m +/+Lepr\(^{db}\)/J) were used for these experiments at a mean age of 12 weeks. All animals were purchased from The Jackson Laboratory (Bar Harbor, ME), and had the same genetic background.\(^{249}\) The animal experiment protocols were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. All animal experiments were conducted under the guidance on humane use and care of laboratory animals for biomedical research published by the National Institutes of Health (No. 85-23, Revised 1996).

Specific Aim #1

The \( db/db \) mice were randomized to receive daily TAD (1 mg/Kg), HO-3867 (25 mg/Kg) [kindly provided by Dr. Periannan Kuppusamy, Ohio State University], a combination of TAD and HO-3867, or control (an equal volume of 10% DMSO) for a total duration period of up to 12 weeks. Each had metabolic parameters monitored such as body weight and fasting glucose, and plasma samples taken for evaluating inflammation (\( n=6-8 \)/group). Eight \( db/db \) mice (\( n=2 \)/group) were used to measure
oxidative stress and isolated cardiomyocytes from the latter groups were used to evaluate for ROS formation and preservation of $\Delta \psi_m$ after 40 min SI and either 1h or 18 h of RO (figure 21).

Figure 21. Experimental protocol
Metabolic monitoring

Each mouse had body weight and 12 h fasting blood glucose level at the start of all experiments and then weekly for the mean 12-week treatment period. Food and water intake was monitored throughout the study.

Insulin and Glucose Tolerance tests

Blood glucose concentrations were obtained using a handheld glucometer (Lifescan, Milpitas, CA). After 10 weeks of treatment, a glucose tolerance tests was performed after 12 h of fasting. After initial blood glucose determinations, 1.5 g/Kg of d-glucose was administered, followed by glucose determinations at 30 min intervals for 120 min. For the insulin tolerance test, human regular insulin (1.5 U/Kg) was injected into random-fed mice and tail vein samples for blood glucose levels were taken at 30 min intervals for 120 min.

Measurement of Cytokines, Chemokines and Triglyceride levels

At the time of sacrifice, each mouse had blood collected by cardiac puncture into 2 separate ethylenediaminetetraacetic acid (EDTA) tubes, stored immediately on ice and centrifuged at 4°C, 3,000 g for 10 minutes. The serum and plasma were separated
and stored at -80°C until analyzed. Plasma concentrations of representative cytokines: IL-1α, IL-1β, IL-2, IL-3, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17, TNF-α, interferon (IFN)-γ and chemokines: RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted) also known as CCL (C-C chemokine ligand)-5, MIP-1α and -1β (macrophage inflammatory protein) which are also known as CCL-3 and CCL-4, Eotaxin (CCL-11 family), was quantified using the Bio-Plex Pro magnetic cytokine assay (Bio-Rad, Hercules, CA) in the initial set of hypothesis generating experiments with TAD [70]. Subsequently, using the same Bio-Plex magnetic cytokine assay, all groups had cytokine and chemokine measurements of IL-1β, IL-6, IL-10, IFN-γ, MCP-1, MIP-1α and -1β, TNF-α, and RANTES.

**Langendorff Isolated Perfused Heart**

The methods for the isolated, perfused mouse heart preparation have been previously described in detail. In brief, each mouse (from each of the treatment groups) was anesthetized with pentobarbital sodium (100 mg/Kg) and the heart was quickly removed from the thorax and placed in a small dish containing ice-cold perfusate and heparin. In this case, heparin was not used in the peripheral circulation as its use would prevent miR analysis from the plasma at a later date. The aortic opening was rapidly cannulated and tied on a 20-gauge blunt needle that was connected to a Langendorff perfusion system. After cannulation, the heart was retrogradely perfused at
a constant pressure of 75 mm Hg with modified Krebs–Henseleit (K–H) solution containing (in mM) 115 NaCl, 4.0 KCl, 2.0 CaCl₂, 25 NaHCO₃, 1.1 MgSO₄·H₂O, 0.9 KH₂PO₄, and 5.5 glucose. The perfusion solution was continuously gassed with 95% O₂ + 5% CO₂ (pH ~ 7.4) and warmed by a heating/cooling bath. The heart temperature was continuously monitored and maintained at 37 °C throughout the experiment. Ventricular function was measured by a force-displacement transducer (model FT03, Grass) attached to the ventricular apex with a no. 5 surgical thread and a rigid metal hook. The resting tension of the isolated heart was adjusted to approximately 0.30 g. Ventricular developed force was continuously recorded with a PowerLab 8SP computerized data acquisition system connected to the force transducer (AD Instruments, Colorado Springs, CO) after daily calibration. Coronary effluent was collected by timed collection of the perfusate to determine flow rate. The hearts were not paced. All hearts were subjected to 20 min of stabilization, followed by 30 min of ischemia and 1 h of reperfusion. After the 1 h reperfusion period, hearts were collected, frozen at -20° C for infarct size assessment 24-48 h later using triphenyltetrazolium (TTC) staining method (figure 22).
**Experimental Protocol – *In Vivo* I/R Model**

**Figure 22.** Langendorff isolated perfused heart protocol.

**Isolation of Ventricular Cardiomyocytes**

Adult male leptin receptor null, homozygous *db/db* mice (strain B6.Cg-m +/Lepr\(^{db}/\)J),\(^{249}\) were used to isolate ventricular cardiomyocytes. The ventricular cardiomyocytes were isolated using an enzymatic technique modified from the previously reported method.\(^{249-251}\) In brief, the animal was anesthetized with
pentobarbital sodium (100 mg/Kg, *i.p.*) and the heart was quickly removed. Within 3 min, the aortic opening was cannulated onto a Langendorff perfusion system and the heart was retrogradely perfused (37 °C) at a constant pressure of 55 mmHg for approximately 5 min with a Ca\(^{2+}\)-free bicarbonate-based buffer containing (in mM): 120 NaCl, 5.4 KCl, 1.2 MgSO\(_4\), 1.2 NaH\(_2\)PO\(_4\), 5.6 glucose, 20 NaHCO\(_3\), 10 2, 3-butanedione monoxime, and 5 taurine, which was continuously bubbled with 95% O\(_2\) + 5% CO\(_2\). The enzymatic digestion was commenced by adding collagenase type II (Worthington, 0.5 mg/mL each) and protease type XIV (0.02 mg/mL) to the perfusion buffer and continued for \(~\)15 min. Fifty µM Ca\(^{2+}\) was then added to perfuse the heart for another 10–15 min. The digested ventricular tissue was cut into chunks and gently aspirated with a transfer pipette for facilitating the cell dissociation. The cell pellet was resuspended for a 3-step Ca\(^{2+}\) restoration procedure (i.e., 125, 250, 500 µM Ca\(^{2+}\)). The freshly isolated cardiomyocytes were then suspended in minimal essential medium (Sigma catalogue# 6 M1018, pH 7.35–7.45) containing 1.2 mM Ca\(^{2+}\), 12 mM NaHCO\(_3\), 2.5% fetal bovine serum, and 1% penicillin–streptomycin. The cells were then plated onto the 35 mm cell culture dishes, which were pre-coated with 20 µg/ml mouse laminin in PBS + 1% penicillin–streptomycin for 1 h. The cardiomyocytes were cultured in the presence of 5% CO\(_2\) for 1 h in a humidified incubator at 37 °C, which allowed cardiomyocytes to attach to the dish surface prior to the experimental protocol.
Measurement of Reactive Oxygen Species

Isolated myocytes were plated onto 96 well microplate and subjected to 40 min SI and 18 h RO. After plating, they were incubated with 50 µM of dichlorodihydrofluorescein diacetate (H₂DCFDA) in growth medium for 30 min at 37°C. Cardiomyocytes were rinsed with PBS, and ROS levels were determined using a fluorescence microplate reader at an excitation of 485 nm and emission of 538 nm.

Measurement of Mitochondrial Membrane Potential (Δψm)

Isolated ventricular cardiomyocytes subjected to 40 min simulated ischemia and 1h reoxygenation were stained with 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazole-carbocyanide iodine (JC-1; Biocarta) by incubating with 2 µg/mL JC-1 for 10 minutes at 37°C. Fluorescence was analyzed with a Texas red–FITC filter cube using a Nikon Eclipse Ti inverted research microscope (Melville, NY). The ratio of mitochondrial aggregates (red) to the monomeric form of JC-1 (green) was analyzed with the use of Q-Capture Professional image analysis software (QImaging). Myocytes were counted from 8-10 separate fields per group and expressed as the ratio of mitochondrial aggregates to monomeric form of JC-1.
Western Blot

The mouse heart samples were collected and the proteins were extracted in a buffer containing (in mmol/L): 50 potassium phosphate, 1 EDTA, 1 EGTA, 0.2 PMSF, 5 beta-glycerophosphate, 2 NaF, 2 Na$_3$VO$_4$, 10 β-mercaptoethanol, 1μg/ml pepstatin, and 0.5 μg/ml leupeptin, (pH 7.0) with a tissue homogenizer. The homogenate was centrifuged at 10,000 g for 15 min under 4°C and then supernatant was recovered. Fifty milligrams of protein from each sample were separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membrane was incubated with primary antibodies at a dilution of 1:1000 for each of the respective proteins, i.e. [PKG, actin (goat polyclonal), phosphorylated Akt (pAkt), Akt, pAMPK, AMPK, (rabbit polyclonal), pCav-1, and Cav-1 (Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was then washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:2000 dilution, 1h at room temperature). Detection of the signals was performed using LumiPhos™ reagent (Pierce) and chemical luminescence was detected using X-omat Kodak film. The densitometry quantification was performed with Bioquant image analysis software.

Evaluation of Cell Viability and Apoptosis

Cell viability was assessed by trypan blue exclusion assay in the cell medium. At the end of protocol, 20 μL of 0.4% trypan blue (Sigma-Aldrich) was added into the
culture dish. After approximately 5 min of equilibration, the cells were counted under a Nikon Eclipse Ti inverted research microscope (Melville, NY). Cardiomyocyte apoptosis for the preliminary experiments was evaluated by using the ApoAlert DNA Fragmentation kit (ClonTech, Mountain View, CA) according to manufacturer’s specifications. The quantification of apoptosis was determined by counting the TUNEL-positive myocyte nuclei from a mean of seven random fields per section and was expressed as percentage of total myocyte nuclei, as previously reported.40

**Infarct size assessment**

At the end of reperfusion, the heart was immediately removed from the Langendorff apparatus and frozen at -20 °C for 24-48 h. The frozen heart was cut into six to seven transverse slices, stained by 10% TTC for 30 min at room temperature (~22 °C), and subsequently fixed with 10% formalin for 24 h. The infarct area was determined by computer morphometry by using ImageJ Software (National Institutes of Health, Bethesda, MD). The infarct size was presented as percentage of the total myocardium (at risk myocardium).
Protein Kinase G Activity

PKG activity was examined after the preliminary hypothesis generating experiments using a commercially available PKG activity kit (Cyclex; MBL International, Japan) in ventricular cardiomyocytes isolated from each group (Activity was measured according to the manufacturer's instructions. Spectrophotometric absorbance was measured at 450 nm. Results were normalized as per milligram of protein.

SPECIFIC AIM #2

In recent years miRs are being increasingly recognized as potential therapeutic targets for a variety of diseases in addition to possible biological markers for making early diagnoses.\textsuperscript{218} MiR-103/107 was recently shown to be upregulated in obese mice and impair glucose tolerance and insulin sensitivity.\textsuperscript{219} Moreover, it is suggested that Cav-1, which is a critical regulator of the insulin receptor, is the direct target gene of miR-103/107. Likewise, given that PDE-5 inhibitors effects eNOS expression and since eNOS temporally and spatially regulates caveolar microdomains that facilitate signal transduction, we propose that TAD would decrease the myocardial expression of miR-103 and -107 and that this may be one of the potential mechanisms by which TAD effects glucose regulation in diabetic mice. Furthermore, to confirm our findings, we will evaluate the expression of its downstream target gene, Cav-1.
Experimental Design

Mice were randomized to receive TAD (1 mg/Kg) or 10% DMSO for a mean of 5 weeks. A third group of non-diabetic mice C57BL/6J mice (n=3) also received vehicle (DMSO). At the end of the treatment period, hearts were extracted to determine myocardial expression of microRNA-103/107, mRNA expression of Cav-1 and protein expression by Western blot analysis of Cav-1.

MiRNA extraction and verification

Total RNA including miRNA was isolated using miRNeasy Mini kit (Qiagen Sciences, MD, USA) according to manufacturer’s protocol. The concentration of RNA was measured using Agilent NanoDrop ND-1000 Spectrophotometer (Houston, Texas, USA). Complementary DNA (cDNA) was synthesized from either 10 ng or 2ug of total RNA using microRNA specific primer (TaqMan miRNA reverse-transcription kit, Applied Biosystems, USA) for microRNA profile and hexamer for mRNA expression (High capacity cDNA synthesis kit (Applied Biosystems, USA).

Real-time qPCR

Real-time PCR was performed on an Applied Biosystems 7900HT Real-Time PCR System (Forest City, CA) using amplicon specific TaqMan microRNA or Gene
expression assay probes. The following are the details of the primer used: For the expression of microRNA 103/107 hsa-miR-103 – AGCAGCAUUGUACAGGGCUAUGA and hsa-miR-107-AGCAGCAUUGUACAGGGCUAUCA were used, respectively, and were normalized using small nucleolar RNA -202 (sno-202). Gene expression quantification for caveolin-1 mRNA expression was carried out using exon spanning primers and an amplicon specific gene assay probe. The mRNA expression of caveolin-1 was normalized using GAPDH as the housekeeping gene. The results were analyzed both by delta-delta cT and absolute quantification using a standard curve method.

**Western blot**

The mouse heart samples were collected and the proteins were extracted in a buffer containing (in mmol/L): 50 potassium phosphate 1 EDTA, 1 EGTA, 0.2 PMSF, 5 beta-glycerophosphate, 2 NaF, 2 Na₃VO₄ 10 β-mercaptoethanol, 1µg/ml pepstatin, and 0.5 µg/ml leupeptin, (pH 7.0) with a tissue homogenizer. The homogenate was centrifuged at 10,000 g for 15 min under 4°C and then supernatant was recovered. Fifty milligrams of protein from each sample were separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membrane was incubated with primary antibodies at a dilution of 1:1000 for each of the respective proteins, i.e. [actin (goat polyclonal), Cav-1 (N-20) - sc-894, Santa Cruz biotechnology, CA, USA].
The membrane was then washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:2000 dilution, 1 h at room temperature). Detection of the signals was performed using LumiPhos™ reagent (Pierce) and chemical luminescence was detected using X-omat Kodak film. The densitometry quantification was performed with Bioquant image analysis software.
CHAPTER 4

STATISTICS

Continuous variables are expressed as mean ± standard error. Two-way analysis of variance (ANOVA) was used to compare pre- and post-intervention values between the 2 groups. Student’s T test was used for comparison of unpaired data between 2 groups and the one-way ANOVA to compare unpaired data between 3 or more groups followed by a Tukey or Bonferroni post-hoc analysis (Prism 5, GraphPad Software, LaJolla, CA). Discrete variables are expressed as percentage and the Chi-square or Fisher’s exact tests are used accordingly. Unadjusted two-tailed P values <0.05 are considered statistically significant.
CHAPTER 5

EXPERIMENTAL LIMITATIONS

The initial study design had experiments that included in vivo I/R protocols to simulate clinical scenarios and then analyze cardiac function by TTE after reperfusion. However, during our preliminary work we discovered that the db/db mouse is especially prone to mortality in the first 24 h after MI. This made post-MI follow-up extremely difficult albeit the ideal scenario for I/R injury. We abandoned this plan and decided to perform an ex vivo isolated perfused Langendorff model of I/R. This allowed us to assess cardiac function before and after ischemia as well as determine infarct size although in an ex vivo model.

A second limitation was that given the large number of animals required for the study and that only 2-3 mouse hearts could be isolated for Langendorff per day, not all mice finished the study in the same week. This gave us a mean completion time of 11 weeks 5 days for all mice. Hence for all results, we selected an end or completion time of “12” weeks. Interestingly, the study was proposed to be 8 weeks in length but temporary limitations in resources, availability of materials, staff, etc., it was decided to expand the length of treatment to 12 full weeks.
During the cytokine analysis not all plasma sample values determined by the cytokine magnetic assay could be used. One or more were reported below the standard concentration curve and were not reported by the system. However, a mean of 4 per group was utilized in calculation.

Utilizing a group of age-matched non-diabetic controls for all experiments throughout the 12 weeks would have been ideal. Instead only non-treated diabetic mice served as controls for most experiments. Having a non-treated, non-diabetic control would have provided valuable additional data and will be utilized for future experiments.

Finally, in the isolated cardiomyocytes experiments, myocytes were isolated from each group using one mouse per group. Although the results we found were significant and comparable to results we have previously published, we would need to repeat this portion of the aim with a larger “n” number. This was limited in part due to the number of mice and the ability to carry out the isolation of more myocytes in the prescribed treatment period without altering the “end time” for each group.
CHAPTER 6

RESULTS

The effects of TAD and HO-3867 on the basic metabolic characteristics of the diabetic mouse

The health status of each mouse was evaluated in terms of body weight and fasting blood glucose over the 12-week period as shown in Table 1 and 2. The diabetic mouse showed significant increases in bodyweight and fasting glucose levels over the 12-week period, however, each of the three treatment regimens: TAD alone, TAD with HO-3867 and HO-3867 alone, all lead to statistically significant reductions in fasting glucose levels with significant improvements in both insulin and glucose tolerance tests. The most robust improvements were seen in the treatment groups that included HO-3867.

However, by the end of the 12-weeks of treatment, mean body weight was reduced with HO-3867 and combination therapy but not TAD when compared to control (61.6±2.1g, 57.0±1.19g, 57.0±1.5g, vs. 65.5±1.5g, respectively [Figure 26]; P<0.05 for HO-3867 and combination groups vs. control). Both HO-3867 and combination treatment led to a dramatic decrease in body weight and fasting glucose, whereas TAD
therapy only led to improvements in glucose and not body weight. This is congruent with our preliminary study using TAD in diabetic mice (Figures 10-11) in which we were unable to show significant weight loss but did demonstrate slight improvements in fasting glucose levels over 4 weeks of treatment.\textsuperscript{244}

Figure 23. Mean fasting blood glucose levels of each group at the start of treatment.
As shown in figures 23 and 24, at the start of the study there was no significant difference between the groups in terms of mean body weight or fasting glucose levels.

![Mean Weight at week 0](chart.png)

*p=ns between all groups

**Figure 24.** Mean body weight of each group at the start of treatment.

Similar to fasting glucose levels, there was no significant difference in mean body weight between the 4 groups at the start of the study.
Table 1. Mean weights over the 12 week treatment period

<table>
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<th>Control</th>
<th>TAD</th>
<th>Combo</th>
<th>HO-3867</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>weight</td>
<td>standard</td>
<td>weight</td>
<td>standard</td>
</tr>
<tr>
<td></td>
<td>error</td>
<td>n</td>
<td>error</td>
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<td>58.60</td>
<td>±1.74</td>
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<tr>
<td>Week 2</td>
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<tr>
<td>Week 3</td>
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<tr>
<td>Week 4</td>
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<td>Week 7</td>
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Table 1 above shows the mean± standard error for each group during the 12 week course of treatment. During weeks 11 and 12, the “n” decreased in each group as we began the isolated perfused heart protocol and collected plasma samples for cytokine assay. The mean weight of the C57BL/6J mouse, which has the genetic background for the leptin receptor null mouse, is around 30 grams.
<table>
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<th>Combo</th>
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<td>n</td>
<td>Fasting Glucose</td>
<td>Standard error</td>
<td>n</td>
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<td>501.30 ±113.40</td>
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<td>4</td>
<td>444.50 ±74.58</td>
<td>4</td>
<td>449.30 ±50.00</td>
<td>8</td>
<td>442.20 ±47.30</td>
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<tr>
<td>Week 3</td>
<td>491.00 ±64.02</td>
<td>4</td>
<td>461.70 ±89.47</td>
<td>4</td>
<td>421.30 ±44.00</td>
<td>8</td>
<td>408.80 ±47.69</td>
</tr>
<tr>
<td>Week 4</td>
<td>459.40 ±60.86</td>
<td>4</td>
<td>422.40 ±59.74</td>
<td>4</td>
<td>399.70 ±32.00</td>
<td>8</td>
<td>391.40 ±22.33</td>
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<tr>
<td>Week 5</td>
<td>449.30 ±60.65</td>
<td>4</td>
<td>434.90 ±67.47</td>
<td>4</td>
<td>346.00 ±25.00</td>
<td>8</td>
<td>349.90 ±19.61</td>
</tr>
<tr>
<td>Week 6</td>
<td>474.23 ±62.96</td>
<td>4</td>
<td>401.30 ±46.42</td>
<td>4</td>
<td>321.80 ±37.00</td>
<td>8</td>
<td>332.70 ±38.37</td>
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<tr>
<td>Week 7</td>
<td>464.20 ±47.42</td>
<td>4</td>
<td>383.20 ±37.39</td>
<td>4</td>
<td>293.20 ±29.30</td>
<td>8</td>
<td>302.40 ±34.32</td>
</tr>
<tr>
<td>Week 8</td>
<td>452.60 ±15.56</td>
<td>4</td>
<td>377.00 ±25.66</td>
<td>4</td>
<td>280.10 ±20.19</td>
<td>8</td>
<td>283.30 ±21.96</td>
</tr>
<tr>
<td>Week 9</td>
<td>458.40 ±29.18</td>
<td>4</td>
<td>377.30 ±36.22</td>
<td>4</td>
<td>281.00 ±19.00</td>
<td>8</td>
<td>284.40 ±30.81</td>
</tr>
<tr>
<td>Week 10</td>
<td>439.90 ±43.11</td>
<td>3</td>
<td>361.00 ±28.12</td>
<td>3</td>
<td>271.00 ±23.16</td>
<td>6</td>
<td>279.20 ±27.41</td>
</tr>
<tr>
<td>Week 11</td>
<td>452.70 ±36.18</td>
<td>3</td>
<td>349.74 ±24.23</td>
<td>3</td>
<td>261.98 ±26.44</td>
<td>5</td>
<td>266.90 ±26.64</td>
</tr>
<tr>
<td>Week 12</td>
<td>432.20 ±37.39</td>
<td>2</td>
<td>359.11 ±31.42</td>
<td>2</td>
<td>278.40 ±27.56</td>
<td>2</td>
<td>253.10 ±29.45</td>
</tr>
</tbody>
</table>

**Table 2. Mean glucose levels over the 12-week treatment period**

Table 2 above shows the weekly fasting glucose levels (mean ± standard error) for each group during the 12-week course of treatment. During weeks 11 and 12, the “n” decreased in each group as we began the isolated perfused heart protocol and collected plasma samples for cytokine assay.
Mean fasting blood glucose values at the end of the study reveal that treatment with HO-3867 and TAD together provided the largest benefit in glucose reduction although the benefit of combination therapy did not provide any added benefit than either compound given individually. Similarly, drugs individually also led to statistically significant reductions in glucose levels.
Figure 26. Mean weight of each group at the end of treatment.

Figure 26 depicts the mean weight of each group at the end of the 12 week treatment period. There was a significant reduction of weight in both the HO-3867 and combination treatment arms, however treatment with TAD alone did not significantly alter weight, albeit there was a trend towards reduction.
In Figure 27, mean fasting glucose levels in each treatment group are plotted over the entire treatment period. There was no significant difference in the initial levels and by week 5, treatment with HO-3867 and combination of TAD and HO-3867 show a statistically significant difference in glucose levels that persist until the end of the study. Likewise, TAD alone also improved glucose readings when compared to control over the treatment period after approximately the 6th week of treatment.
Figure 28. Mean body weight of each group plotted over the entire treatment period.

The mean body weight in each treatment group gradually increased over the 12 week treatment period with the exception of groups being treated with HO-3867. At approximately week 5, those two groups started to have decline from their mean starting weight, however at the end of the study period the starting and ending weights were nearly the same. Both the TAD and control groups had significant increases in weight during the 12-week period, although net weight gain was less in the TAD treated mice, this did not reach statistical significance.
During the insulin tolerance test, random fed mice were fasted for 6 hours, given 1.5 Units/Kg of regular insulin after baseline reading and then had blood glucose levels monitored over 120 min. Both HO-3867 and combination treated groups had improved insulin sensitivity with enhanced glucose homeostasis at 30 min onwards when compared to control. At 60 min and 90 min, both HO-3867 containing groups had improvements that were statistically significant when compared to TAD as well. Treatment with TAD alone led to only one point in time, at 30 min, in which insulin tolerance was statistically better than control.
There was a notable improvement in glucose tolerance in the treatment groups vs. control. This demonstrates the improved ability of the treated db/db mice to clear circulating glucose and utilize it for oxidation. We were able to show that at all time points a significant difference existed between TAD, HO-3867 and combination groups vs. control, however no significant difference between the groups was found.

**TAD, HO-3867, and combination therapy protects against global I/R Injury**

After 12 weeks of chronic therapy with TAD, HO-3867 and combination treatment we found that following global I/R injury, myocardial infarct size (mean ±SEM) was
greater in the *db/db* mice treated with control (DMSO). Control mice had an infarct size of 43.14±10.5% vs. TAD, which had 20.7±6.0%, HO-3867 of 28.4±8.4% and combination therapy of 25.2±11.1%; p<0.05 for each group vs. control; Figure 31.

![Figure 31. Infarct size after I/R injury.](image)

A total of 4-6 diabetic mice from each group were subjected to I/R injury in a Langendorff isolated perfused heart model. After 30 min ischemia and 60 min reperfusion, the mean infarct sizes in TAD, HO-3867 and the combination group were significantly smaller than in the control group.
The subsequent photographs (Figures 32-35) depict one example of the gross cardiac pathology after global I/R injury using the Langendorff isolated perfused heart model from each of the treatment arms.

Figure 32. TTC stained sections of myocardium after I/R injury from a control (DMSO) treated mouse.

After I/R on the Langendorff apparatus, hearts were collected and immediately frozen at -20° C overnight. The following day, each heart was cut into 6-7 transverse
sections and stained with TTC for 30 min and then fixed with 10% formalin overnight. Using ImageJ software (National Institutes of Health, Bethesda, MD), we measured infarct size as the % of the total myocardium.

Figure 33. TTC stained sections of myocardium after I/R injury from a HO-3867 treated mouse.

Infarcted myocardium is depicted as white/beige tissue whereas healthy, viable myocardium is surrounding the infarcted area and is red/pink in color.
After a 20 min stabilization period, each heart was subjected 30 min ischemia followed by 60 min of reperfusion. Hearts preconditioned with TAD, HO-3867 or the combination of the two had significantly smaller infarct sizes when compared to the control treated mice.
Cardiac Hemodynamics and Contractile Force

Coronary flow rates did improve in the HO-3867 and TAD treatment groups as the % change in coronary flow compared to the pre-ischemia baseline when compared to control. As the percentage of pre-ischemia baseline, the TAD group was
130.2±16.2%; HO-3867 was 132.2±8.0%; 116.5±7.1% for combination treated and 96.7±5.7% in the DMSO treated control mice, (p<0.05 for all groups vs. control; Figure 36). There was no significant difference in the pre-ischemia basal functional parameters (i.e., developed force, rate-force product, and resting tension) between the treatment groups (n=4-6/group). There was no significant change in post-ischemic rate-force product as the % of pre-ischemic baseline compared to the control group with TAD +/- HO-3867 treatment [63.9±8.6% (TAD alone), 31.9±12.3% (HO-3867 alone) and 39.7±12.9% for combination therapy vs. 47.7±4.8% for control; Figure 37].

Similarly, the combination treatment did not lead to any improvement rate-force product when compared to control. Table 3 shows the baseline cardiac functional parameters for each of the treatment groups.

**Table 3. Baseline Cardiac Functional Parameters**

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>TAD</th>
<th>HO-3867</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>310.0±60</td>
<td>350.0±14.1</td>
<td>430.0±36.7</td>
<td>388.3±38.3</td>
</tr>
<tr>
<td>DF (g)</td>
<td>0.97±0.11</td>
<td>1.16±0.7</td>
<td>0.94±0.16</td>
<td>1.01±0.25</td>
</tr>
<tr>
<td>RFP (g beats/min)</td>
<td>583.5±142.43</td>
<td>814±171</td>
<td>802.1±152.2</td>
<td>605.4±194.6</td>
</tr>
<tr>
<td>CF (mL/min)</td>
<td>2.2±0.2</td>
<td>2.4±0.3</td>
<td>2.3±0.2</td>
<td>2.1±0.2</td>
</tr>
</tbody>
</table>

Mean ± SE; HR- heart rate; DF- developed force; RFP- rate force product; CF – coronary flow; g- grams;
Coronary effluent was collected over time during the stabilization period and then during reperfusion during each I/R heart protocol. Coronary flow rates (as the % of pre-ischemia baseline) were improved in each of the treatment arms when compared to control.
The cardiac function is presented as the double product of the heart rate and ventricular developed force (% of the pre-ischemic baseline). There were no significant changes between the groups in terms of cardiac function.
**TAD, HO-3867 and Combination treatment attenuates oxidative stress induced by diabetes**

Diabetes is usually accompanied by increased production of free radicals and impaired antioxidant mechanisms. Hence we measured ROS generation by staining isolated ventricular cardiomyocytes from each treatment group with indicator dye H$_2$DCFDA after 1 h simulated ischemia and 18 h reoxygenation. ROS formation was significantly increased in the *db/db* control group (DMSO) following SI/RO. TAD, HO-3867 and combination treatment all significantly attenuated ROS generation in *db/db* mice as shown in Figure 38.

**ROS Generation after Simulated Ischemia/Reoxygenation**

After 40 min SI and 18 RO, isolated cardiomyocytes from each of the treatment groups were subjected to 10 µM H$_2$DCFDA staining and then ROS generation was determined as % of total control by fluorescence using a microplate reader (excitation of 485 nm and emission of 538 nm). Despite our preliminary data showing a significant decrease in ROS production after chronic TAD treatment, we only found a decrease in ROS after HO-3867 and combination HO-3867 with TAD therapy (Figure 38).
In the diabetic, ROS are involved in insulin resistance via its regulatory effects on mitochondrial function.\textsuperscript{252} Therefore we measured dissipation of $\Delta\psi_m$ of isolated ventricular cardiomyocytes following 40 min SI and 1 h RO by JC-1 staining. Cardiomyocytes from DMSO-treated diabetic mice exhibited a significant loss of $\Delta\psi_m$.
while myocytes isolated from those treated with TAD and/or HO-3867 demonstrated preserved Δψm and intact mitochondrial membranes (Figures 39-43). Hence, these drugs might preserve Δψm in diabetic hearts, at least in part, via mitochondrial activation.

Figure 39. Ratio of JC-1 aggregate:monomer after SI/RO.

* p < 0.05 vs. control

^ p < 0.05 vs. combo
Isolated cardiomyocytes from DMSO (control) treated mice showed loss of the mitochondrial membrane potential after SI/RO after JC-1 staining. More JC-1 exists in monomer form than aggregates as the mitochondria have lost the positive motive force required for oxidative phosphorylation.
Figure 41. JC-1 staining of cardiomyocytes from combo treated mice.

Figure 41, shows JC-1 stained cardiomyocytes after SI/RO, and JC-1 stain has mostly formed aggregates within the inner mitochondrial membrane given maintenance of the mitochondrial membrane potential.
Figure 42. JC-1 staining of cardiomyocytes from HO-3867 treated mice.

After 12 weeks of treatment with TAD, cardiomyocytes subjected to SI/RO, showed a much higher level of JC-1 in aggregates when compared to myocytes isolated from control hearts.
Figure 43. JC-1 staining of isolated myocytes from TAD treated mice after SI/RO

As seen in Figures 40-43, isolated cardiomyocytes were subjected to 40 min SI and 1 h RO and then stained with JC-1 to evaluate for preservation of the mitochondrial membrane potential. Isolated cardiomyocytes from every treatment arm showed preservation of the $\Delta \psi_m$ by aggregate formation of JC-1 within the mitochondria as indicated by orange-red fluorescence. Control cardiomyocytes subjected to similar time period of SI and RO showed significant loss of the $\Delta \psi_m$ by a decrease in JC-1
aggregates (less accumulation within the inner membrane) and higher levels of JC-1 in monomer form as indicated by green fluorescence. Figure 39 shows the ratio of JC-1 of aggregate to monomer formation for each of the treatment groups. With maintenance of the membrane potential, the myocytes are still capable of oxidative phosphorylation and are less likely to create ROS.

**Role of AMPK-Akt signaling in Cardioprotection induced by TAD**

To confirm the NO-AMPK-Akt signaling pathway induction by TAD, myocardial protein levels of AMPK and Akt were assessed by Western blot analysis. Western blot showed that the phosphorylated forms of Akt and AMPK in db/db mice hearts were significantly lower than in non-diabetic C57BLKS/J control mice (n=3). Chronic treatment with TAD significantly enhanced phosphorylated Akt and phosphorylated AMPK, suggesting that TAD treatment increases NO-AMPK-Akt signaling in the diabetic hearts (Figure 44-45) and improved signaling through these pathways may be one of mechanisms for cardioprotection and increased glucose sensitivity.
Figure 44. Western blot and densitometric results of Akt and AMPK.

The figure above illustrates the decrease in phosphorylated Akt to total Akt expression in the diabetic mouse, which was restored after chronic TAD therapy. Likewise, the diabetic mouse has lower myocardial levels of phosphorylated AMPK, which was also restored to physiological levels after chronic TAD treatment, as shown in the lower half of the Western blot. This may be one of the mechanisms by which TAD provides cardioprotection in diabetic mice and further augments the insulin signaling pathway.
The densitometry results show that the ratio of phosphorylated AMPK to total AMPK was significantly increased after chronic TAD therapy. The normal diabetic animal has been shown to have decreased levels of activated AMPK and reduced signaling via the critical metabolic regulator, AMPK. Treatment with TAD was able to restore AMPK in db/db mice by increasing activated AMPK to levels normally found in non-diabetic mice.

**Cardiomyocyte Necrosis**

Our method of cell preparation yielded at least 90% of the isolated cardiomyocytes with rod shape morphology. After 40 min of SI and 1 h of RO, the percent of trypan blue-positive (necrotic) cardiomyocytes was 79.8±1.5% in the control (DMSO) group. Treatment with TAD, HO-3867 and combination treatment resulted in a
statistically significant decrease in the number of trypan-blue positive cardiomyocytes. The number of trypan-blue positive cells expressed as the % of total cells for each group was: 44.2±4.9% (TAD), 27.3±3.2% (HO-3867) and 34.9±3.9% (combination treatment), respectively; p<0.05 vs. 73.5±3.6% (control) for each group (Figure 46). In addition, we found a significant decrease of necrotic myocytes between the HO-3867 treatment group and the TAD group as well (p<0.05).
The total number of trypan-blue positive cells was significantly reduced after chronic therapy with TAD, HO-3867 and combination therapy. Surprisingly, HO-3867 provided the most potent benefit in preventing necrosis after 40 min SI and 1 h RO whereas combining TAD and HO-3867 did not provide any synergistic benefit. However, the possibility of any additive benefit cannot be totally excluded. The synthetic curcumin analogue, HO-3867 not only had a significant reduction when compared to control, but also when used alone it had statistically significant reduction when compared to TAD alone. Hence, not seeing a dramatic reduction or statically significant reduction between combination therapy and TAD or HO-3867 alone, the difference seen with HO-3867 and the significant decline with combination therapy (vs. control) does provide some evidence that an additive benefit may exist. Future experiments using several mice for cardiomyocyte isolation may provide further supportive data.
Figure 47. Trypan-blue staining of cardiomyocytes from control (DMSO) treated mice after SI/RO

Trypan-blue exclusion of myocytes from control treated mice after 40 min SI and 1 h RO reveals a significant number of necrotic cells with a very few number of preserved cells without trypan-blue staining and even fewer with rod-shaped morphology.
Figure 48. Trypan-blue staining of cardiomyocytes from TAD treated mice after SI/RO.

Diabetic mice subjected to chronic TAD treatment and then had hearts isolated for cardiomyocyte isolation and subsequent SI/RO, showed less necrotic cells and increased preservation of myocytes with less trypan-blue positive cells and more cells having a rod-shaped morphology when compared to control.
Isolated cardiomyocytes from mice treated with combination therapy had an increase in cell survival with less trypan-blue positive cells when compared to control.
Figures 47-50, provide quantitative data of the *in vitro* effects of TAD, HO-3867, and combination therapy on isolated ventricular cardiomyocytes following 40 min SI and 1h RO using a Nikon Eclipse Ti microscope (Melville, NY). After 1 h of RO, trypan-blue positive nuclei were counted in respect to the total number of cell nuclei seen. The TAD, HO-3867 and combination treated groups had significantly lower trypan-blue positive nuclei as compared with cardiomyocytes treated with control. In addition, myocytes
isolated from the HO-3867 group were much less necrotic when compared to myocytes isolated from TAD treated mice as well, p<0.05; Figure 46.

**Plasma Inflammatory Cytokine and Chemokine Levels**

There was a significant decrease in nearly all pro-inflammatory cytokines measured after 12 weeks of treatment with TAD, HO-3867 or combination therapy when compared to DMSO with the exception of IL-6 (Figures 51). Surprisingly, there was no decrease in the *anti-inflammatory* cytokine IL-10 in any of the treatment arms when compared to DMSO, (p=ns; Figure 55). There was however a trend towards an increase in IL-10 with combination therapy, but this did not achieve statistical significance.

We found a significant reduction in circulating levels of the pro-inflammatory cytokines TNF-α and IL-1β (Figures 52 and 53). Unfortunately, we did not find any additive or synergistic anti-inflammatory effect when combining the two therapies. Interestingly, we did not see a decrease in circulating IL-6 levels with any of the treatment therapies, in fact there was an *increase* in IL-6 with TAD therapy when compared to control and the other treatment arms (Figure 54). The other treatment groups had no significant decrease in IL-6 levels when compared to control. Currently there is an ongoing debate on whether IL-6 is beneficial or detrimental in diabetes and the use of leptin receptor null mice would only further complicate the cellular and
molecular dynamics. The chemokines MIP-1β (CCL-4), MCP-1 (CCL-2) and RANTES (CCL-5), were all significantly reduced with TAD, HO-3867 and combination therapy when compared with control (Figures 57-59). Plasma levels of the chemokine MIP-1α (CCL-3) however could not be interpreted as all levels measured using the Bio-Plex Magnetic beads assay were below the standardized concentration curve and could not be analysed.

![Graph](image.png)

**Figure 51. Reduction of inflammatory cytokines and chemokines vs. control (DMSO).**

The figure above shows the percent reduction of the eight assessed inflammatory cytokine and chemokines in the 3 treatment groups as compared to the DMSO treated mice. There were notable reductions in the key pro-inflammatory cytokines, however given the increase in IL-6 in the TAD group; it is difficult to appreciate the changes.
Figure 52. Reduction in inflammatory cytokines and chemokines vs. control (DMSO) treated mice.

Figure 52 also shows the percent reduction of the inflammatory cytokine and chemokines in the 3 treatment groups as compared to the DMSO treated mice although with the exclusion of IL-6, the reductions in each group is much easier to appreciate.
After 12 weeks, circulating plasma levels of the inflammatory cytokine TNF-α was significantly reduced in each treatment group. There was a robust decline from control levels 264±12.8 pg/mL vs. 56.6±11.6 pg/mL for TAD, 60.3±10.9 pg/mL in the HO-3867 group and 36.3±6.2 pg/mL for combination therapy (p<0.05 for all groups vs. control). The combination therapy group had the most profound reduction in TNF-α levels, although when compared to TAD and HO-3867 this reduction was not significant.
Plasma levels of the pro-inflammatory cytokine IL-1β were reduced after 12 weeks of treatment in every group when compared to control. The combination group did not have any additive benefit in attenuating IL-1β. The control levels of IL-1β after treatment was 81.7±5.7 pg/mL vs. 39.3±4.5 pg/mL for TAD, 32.7±5.6pg/mL in the HO-3867 group and 40.7±9.1 pg/mL for combination therapy (p<0.05 for all groups vs. control).

Figure 54. Plasma levels of IL-1β.
Interestingly, no treatment group led to a decrease in IL-6 levels although there was not a statistically significant increase in either the HO-3867 (4.6± 1.2 pg/mL) or combination groups (2.8± 0.52 pg/mL) either. There was however, a dramatic increase in IL-6 after chronic TAD treatment (10.4± 3.5 pg/mL) as compared to control (2.6± 0.1 pg/mL).
Plasma levels of anti-inflammatory cytokine IL-10 were not significantly different in the TAD (42.1±13.6 pg/mL) or HO-3867 (44.8±4.1 pg/mL) treatment groups when compared to control (64.6±2.6 pg/mL). We did find a trend towards an increase in IL-10 levels after 12 weeks of combination treatment (71.8±8.0 pg/mL), however this did not reach statistical significance.
IFN-γ

* p<0.05 vs. control

Figure 57. Plasma levels of IFN-γ.

The dimerized soluble cytokine IFN-γ is a key mediator of macrophage activation and plays a significant role in autoinflammatory and autoimmune diseases. It has been shown to be upregulated and associated with obesity-induced inflammation. After 12 weeks of therapy, plasma levels of IFN-γ were dramatically reduced with TAD (2.9±0.7 pg/mL), HO-3867 (2.7±0.1 pg/mL) and combination treatment (3.9±0.5 pg/mL) when compared to control (7.1±0.8 pg/mL), p<0.05 vs. all groups.
The chemokine MIP-1β or chemokine (C-C motif) ligand (CCL-4) is also a macrophage activator and helps recruit macrophages to adipose tissue which perpetuates the pro-inflammatory cascade associated with insulin resistance and obesity. After treatment however, circulating levels of MIP-1β were reduced with TAD (12.0±0.9 pg/mL), HO-3867 (10.9±2.9 pg/mL) and combination treatment (6.9±1.0 pg/mL when compared to control (24.8±2.3pg/mL), p<0.05 vs. all groups.

Figure 58. Plasma levels of MIP-1β.
Monocyte chemoattractant protein-1 (MCP-1), also known as CCL2, is a chemokine that activates monocytes, memory T cells, and dendritic cells to the sites of infection or inflammation. More recently, MCP-1 was shown to induce amylin expression through ERK1/2, JNK-AP1 and NF-κB related signaling pathways independent of the MCP-1 receptor. Amylin upregulation by MCP-1 is known to contribute to the elevation of plasma amylin and insulin resistance in obesity. We found that treatment with each compound significantly reduced levels of MCP-1. Circulating levels after treatment with TAD were 30.5±5.7 pg/mL, HO-3867 (37.3±3.4 pg/mL) and combination treatment (27.2±2.2 pg/mL) when compared to control (61.5±1.8 pg/mL), p<0.05 vs. all groups.

Figure 59. Plasma levels of MCP-1.
Regulated upon and normal T-cell expressed and secreted (RANTES), also known as CCL-5 is a chemokine that plays a significant role in recruiting various leucocytes to inflammatory sites. It is currently unknown what exact role RANTES plays in the obesity-induced inflammatory disorder but given its chemotactic ability, we can hypothesize that attenuation of its production would decrease the inflammatory response. Accordingly, we found treatment with TAD (30.5±5.7 pg/mL), HO-3867 (37.3±3.4 pg/mL) and combination of both (27.1±2.3 pg/mL) all significant reduced circulating levels of RANTES when compared to control (61.5±1.8 pg/mL), p<0.05 vs. each group.

Figure 60. Plasma levels of RANTES.
**The Effects of TAD therapy on MicroRNA -103/107 and Cav-1**

As shown in Figures 60 and 61, *db/db* mice treated with DMSO had a dramatic rise in myocardial miR-103 and miR-107 expression when compared to non-diabetic controls.

![Graph showing miR-103 expression levels.](image)

**Figure 61.** Myocardial miR-103 expression after TAD treatment.
Treatment with TAD caused a significant reduction in miR-103 levels (Figure 60) and miR-107 (Figure 61) as compared to the db/db DMSO-treated control, to levels that were similar to the non-diabetic control. Since the downstream target gene of miR-103/107 has been predicted to be caveolin-1 based on complimentary sequence alignment, we evaluated myocardial mRNA levels of Cav-1 and confirmed this by Western blot analysis.

![Graph showing miR-107 expression after TAD treatment.](image)

Figure 62. Myocardial miR-107 expression after TAD treatment.
The total myocardial mRNA expression of Cav-1 was significantly reduced in the db/db mice, which correlated with the increased expression of myocardial miR-103 and -107. To further confirm the reduced mRNA levels of caveolin-1, we performed a Western blot analysis for protein expression and similarly found lower levels of CAv-1 protein in the diabetic controls. Conversely, after chronic TAD treatment, there was a significant increase in Cav-1 mRNA expression as well as protein levels (Figures 63 and 64).
Given that both miRs negatively regulate Cav-1, we found a statistically significant increase in Cav-1 expression with chronic TAD treatment, which appropriately correlated with the decrease in miR 103/107. Likewise, the db/db group had significantly lower levels of Cav-1, which corresponded with the much higher levels of both miRs (Figures 62-63).

Figure 64. Western blot and densitometry data of Cav-1 protein.
Evaluating total protein expression of Cav-1, we found a significant increase in the non-diabetic controls which significantly downregulated in the diabetic mice. This could have been related to the elevated miR-103 and miR-107 expression since it is speculated that the downstream target gene of miR-103/107 is Cav-1. Chronic TAD therapy attenuated this dramatic decline in Cav-1 levels and this may be one mechanism by which chronic TAD treatment improves insulin signaling as Cav-1 is known to improve insulin receptor stabilization and enhance insulin signaling.
CHAPTER 7

DISCUSSION

For the first time, we have demonstrated that chronic administration of a synthetic analogue of curcumin attenuates inflammation, significantly improves insulin sensitivity, reduces mean body weight and ameliorates infarct size after I/R injury both with and without the concomitant administration of the PDE-5 inhibitor TAD. The treatment regimens of TAD and HO-3867 exhibited beneficial effects on various systemic metabolic abnormalities induced by insulin resistance and obesity and together were effective in improving the metabolic status of obese mice as evidenced by improvements in body weight, fasting blood glucose and both insulin and glucose tolerance tests. The db/db mouse model is a model of severe type II diabetes in morbidly obese animals and we hypothesized that the moderate reductions in terms of body weight and blood glucose observed in our preliminary investigations could be further accelerated and potentiated if TAD treatment was coupled with another anti-hyperglycemic drug. Although clinical studies indicate that the incidence and severity of AMI are greater in the diabetic population compared to the non-diabetic populations, experimental data reveals conflicting data on the vulnerability of the diabetic heart to ischemia in diabetic animal models. These inconsistent results can be partially explained by different durations and severity of diabetes and interspecies
Moreover, the severity and type of ischemic challenge can modify the response of the diabetic heart to injury. In this investigation, we challenged the diabetic hearts to 30 min global ischemia followed by 1 h reperfusion that resulted in increased sensitivity to ischemic insult as evidence by larger infarct sizes in the untreated diabetic mice hearts (Figures 31-35). Cardiac function did not significantly improve in the treatment group during isolated I/R, albeit significant variability between isolated hearts in the same treatment group quite possibly lead to this non-significant finding. We speculate that possible differences at the time of experimentation such as quality of buffer preparation (given the requirement for daily preparation), pH variability, and in our model, external temperature, which could not be stringently regulated to be maintained continuously at 37° C without any variance. Each issue could potentially limit and/or hinder cardiac performance in the isolated perfused heart model. We did however find a difference in coronary flow rates with improvements in coronary flow in the treatment groups when compared to the DMSO treated controls (Figure 36).

Likewise, in vitro studies using isolated cardiomyocytes from each treatment arm yielded a reduction in necrotic cells after chronic administration with TAD both with and without HO-3867 after 40 min SI and 1 h RO. The cardioprotective effects of these drugs, specifically TAD, are consistent with our previous studies that demonstrate that PDE-5 inhibitors can induce powerful cardioprotective effects against in vivo myocardial I/R injury, yet in the normoglycemic mice.
A number of cellular signaling pathways have been demonstrated to be involved in the cardioprotective effects of TAD. Previously we reported that TAD treatment enhances cGMP and PKG levels in murine models of I/R injury indicating that the cardioprotective effect of TAD is through the NO-cGMP-PKG signaling cascade.\textsuperscript{260,261} Likewise, we have shown that other PDE-5 inhibitors, such as sildenafil and vardenafil, upregulate PKGI\textgreek{a}, eNOS/iNOS protein expression, open the mitochondrial $K_{\text{ATP}}$ channel and through phosphorylation and inactivation of GSK-3$\beta$, prevent opening of the mPTP—all of which lead to cardioprotection (Figure 64).\textsuperscript{30, 32, 211, 262, 263}

The phosphorylation and activation of PI3K leads to activation and phosphorylation of Akt, which phosphorylates and inactivates GSK-3$\beta$. We evaluated the myocardial expression of phosphorylated Akt (pAkt) in non-treated $db/db$ controls, non-diabetic controls, and $db/db$ mice treated chronically with TAD. We found a significant decline in phosphorylated Akt in diabetic mice, which was restored after TAD treatment. Similarly, $db/db$ mice are known to have lower than normal levels of activated/phosphorylated AMPK (pAMPK), a key regulator of metabolism, which were significantly lower in the diabetic mouse yet restored to basal non-diabetic levels with TAD therapy. These observations confirm previous studies that demonstrate the critical role of Akt and AMPK in mediating the cardioprotective effects in diabetic hearts.\textsuperscript{264}
PDE-5 inhibitors have also been shown to increase hydrogen sulfide (H₂S) levels through PKG which upregulates the enzyme responsible for H₂S production---CSE. Recent data support a number of beneficial and cardioprotective effects of H₂S. Through increased H₂S levels, downstream effects include opening of the mitochondrial K\text{ATP} channel, increased PKC which decreases intracellular calcium load and prevents opening of the mPTP. Likewise, through PI3K, Akt and PKC-θ signaling cascade it can increase expression of anti-apoptotic proteins, Bcl-2 and Bcl-XL. Another beneficial effect of H₂S is that it is a known activator of Nrf2, which allows its translocation to the nucleus for transcription of antioxidant genes such as heme oxygenase-1 (HO-1), glutathione peroxidase (GPX), catalase, and glutathione-S-transferase (GST) (figure 64).

Oxidative stress mediated by hyperglycemia-induced generation of ROS contributes significantly to the development and progression of diabetes and related vascular damage.\textsuperscript{265} Impaired antioxidant defenses coupled with an increase in ROS, both contribute to oxidative stress and numerous studies have shown that ROS generation increases in both type I and type II diabetes.\textsuperscript{266-269} Moreover, ROS are involved in insulin resistance via its regulatory effects on mitochondrial function.\textsuperscript{270}
Figure 65. Possible mechanisms by which TAD attenuates inflammation, improves glucose levels and offers cardioprotection

The illustration above represents the various pathways that TAD may affect signaling to attenuate inflammation, glucose levels, myocyte apoptosis and necrosis. TAD through inhibition of PDE-5 can increase cGMP leading to increases in phosphorylated PI3K and Akt. This can inactivate GSK-3β by phosphorylation, which will inhibit opening of the mPTP. Likewise, cGMP increase PKG1α levels, which phosphorylates and activates VASP. VASP has been shown to attenuate inflammation by blocking NF-κB. PKG1α also can directly open the mitoK_ATP channel, which further leads to cardioprotection. Through the insulin receptor substrate (IRS) and the increased Akt and PI3K from downstream PDE-5 inhibition, there can be an increase in GLUT4, which promotes glucose uptake.
In the present investigation we demonstrate that chronic treatment with TAD and HO-3867 attenuates oxidative stress induced in an animal model of type II diabetes and likewise decreases the ROS production in isolated ventricular myocytes following SI/RO injury. In addition, increased ROS generation augments impairment of mitochondrial function in diabetic hearts.271

As both pharmacological agents have shown the capability to attenuate ROS generation and reduce oxidative stress, we investigated whether the proposed treatment regimen would preserve the Δψm. Accordingly, JC-1 immunofluorescent staining revealed dissipation and loss of mitochondrial integrity following SI/RO in cardiomyocytes isolated from db/db mice treated with DMSO alone. However, TAD with and without HO-3867 significantly preserved the loss of Δψm. These findings demonstrate that the protective effects of TAD and HO-3867 in diabetes-induced oxidative damage may be due effective ROS scavenging and thus maintaining a delicate balance in oxidant-antioxidant status of the myocardium. This effectively suggests that either therapeutic compound may be a promising drug in avoiding the development of diabetes-induced oxidative cardiotoxicity. In addition, we recently published that chronic treatment with TAD in diabetes improves redox signaling by enhancing the antioxidant enzyme glutathione-S-transferase kappa-1 and downregulates redox regulatory chaperones, HSP8 and 75 kDa glucose regulatory protein.272 One of the proposed mechanisms, as shown in Figure 65, may be through upregulation of hydrogen sulfide producing enzyme, CSE. This effectively increases
H₂S which upregulates Nrf2 and enhances its nuclear translocation. One of the downstream target genes of the Nrf2 transcription activator is glutathione-S-transferase.

Chronic inflammation induces insulin resistance through upregulating metabolic alterations in blood pressure, blood glucose levels, and lipids. Thus, a deleterious cycle begins as adipose tissue release pro-inflammatory cytokines, which in turn leads to the development of insulin resistance and atherogenesis.²²⁴,²⁷³ At the cellular level, nutrient excess has been shown to activate the IKK-β/ NF-κB pathway within adipose tissue, which is associated with the infiltration of macrophages. It is through this mechanism TNF-α and IL-1β both directly impair the insulin-signaling cascade whereas macrophages are the main source of inflammatory mediators within both murine and human adipose tissue. A significant finding advancing the study of obesity-induced inflammation was the discovery that the marked increase in inflammatory adipokine production was directly related to the number of macrophages within the adipose tissue.

We also showed for the first time that circulating levels of key pro-inflammatory cytokines, TNF-α and IL-1β, were significantly reduced after chronic TAD± HO-3867 treatment. However, the combination of the two therapies did not lead to any further reduction in circulating cytokines. This could be arriving at a maximum reduction or attenuation of circulating cytokines achieved and that combination of the two compounds could not lead to any further decrease. On the standard concentration curve, values for both TNF-α and IL-1β were on the lower end and values were often
omitted because they were well below the standard curve. Nevertheless, each compound reduced both inflammatory markers either individually or in combination.

The anti-inflammatory cytokine IL-10 however did not change between the groups, although in our preliminary experiments we saw a slight, but statistically significant, increase in IL-10 after 4 weeks of TAD treatment. Previously published data and known physiological pathways show that IL-10 levels are inversely proportional to adiposity. Adipose tissue of lean mice secretes an unusually high level of IL-1, which suppresses adipose tissue inflammation. Furthermore, the number of T cells that secrete IL-10 dramatically decrease in proportion to increasing obesity. After 12 weeks of therapy, there was not a statistically significant difference in IL-10 levels between the groups. A non-diabetic control group would have been helpful in determining if the levels were higher in a treated group than in the DMSO treated diabetic group. Likewise, performing cytokine analysis both at the start of the study and repeating an analysis at the end could also provide additional data for these conflicting results.

Surprisingly, we found that there was an increase in IL-6 after chronic TAD treatment, whereas there was no difference in IL-6 levels between control and the other treatment groups. This finding is also different than our preliminary findings with TAD and DMSO treatment for 4 weeks. In the preliminary work, there was a small and statistically insignificant decline in IL-6 with TAD therapy. Currently after 12 weeks,
there was a greater increase in circulating IL-6 levels with TAD treatment alone. The combination and HO-3867 groups were no different than control. Currently, the exact role of IL-6 in the pathophysiology of insulin resistance remains controversial and to date there is no consensus although various experiments have provided some critical information on its effects on the insulin signaling cascade. In insulin-resistant individuals, IL-6 levels were found to be significantly elevated in adipose tissue. Moreover, IL-6 acts both centrally and peripherally to induce energy expenditure and impair insulin signaling. However, both deficiency and overexpression of IL-6 lead to severe hepatic inflammation and insulin resistance. In cell culture models, hepatic and adipocyte insulin signaling can be inhibited by IL-6 through IRS-1 inhibition. Future experiments with tissue specific targeting of IL-6 will likely improve our understanding of IL-6 and its pathophysiological role in insulin resistance.

The monocyte chemoattractant protein-1 (MCP-1) is produced by adipose tissue in response to dietary excess and is a key factor in recruiting the macrophage precursor, the monocyte. Studies in MCP-1−/− and mice overexpressing MCP-1 confirm the role of this chemokine in attracting macrophages to adipose tissue and in reducing insulin sensitivity in high-fat diet induced obesity. Mice lacking the receptor for MCP-1 showed a lower macrophage content in visceral adipose tissue and improved insulin sensitivity after high-fat diet feeding. Likewise, the expression of a dominant negative mutant for MCP-1 in obese db/db mice significantly decreased their insulin resistance. In addition to MCP-1, human studies have confirmed the increase of
MCP-2, RANTES, and macrophage inflammatory protein (MIP)-1α in obese individuals when compared to lean cohorts.\textsuperscript{281}

Macrophage inflammatory protein-1β (MIP-1β), MCP-1 and RANTES are three chemokines with the C-C ligand (CCL) motif and play a significant role in the activation and proliferation of the inflammatory cascade, especially in adipose tissue and the endothelium.\textsuperscript{282} Not surprisingly, TNF-α can activate both MCP-1 and MIP-1β which further recruit macrophages to the adipose tissue, hence potentiating the inflammatory response.\textsuperscript{283} This invariably leads to the chronic low-level state of inflammation found in obesity. We found that along with reduced levels of TNF-α, the chemokines MIP-1β, MCP-1 and RANTES were all significantly reduced with all three treatments as compared with control.

Early in obesity-related insulin resistance there is a reduction in vascular NO content, which predisposes to increased vascular inflammation, thrombosis and vasoconstriction. This precedes the increase in hepatic NF-κB signaling and impaired insulin signaling through phosphorylated Akt. Nitric oxide signaling includes activation of PKG and one downstream target is vasodilator-stimulated phosphoprotein (VASP), a protein implicated in the control of cytoskeletal dynamics and cell migration.\textsuperscript{169} Several studies have shown that diet-induced obesity and genetically induced murine models of obesity, both reduce vascular NO content which precedes a dramatic increase in hepatic inflammation and hepatic insulin resistance (decrease in insulin stimulation of
the IRS-1/PI3K/Akt pathway). In addition, the decline in NO content precedes the inflammation, which is characterized by an increase in NF-κB signaling. Several dozen studies have established the role of curcumin in inhibiting NF-κB signaling through directly blocking the HAT p300/CBP. In a study by Handa et al., they found that reduced VASP directly correlated with adipose tissue inflammation (increased TNF-α, IL-6) and was also associated with significantly low levels of NO and phosphorylated eNOS.\textsuperscript{169} In addition, they found that phosphorylation of VASP by PKG was critical in the attenuation of hepatic inflammation and insulin resistance and VASP\textsuperscript{-/-} mice were especially vulnerable. Moreover, restoration and/or augmentation of VASP signaling with use of a PDE-5 inhibitor attenuated insulin resistance, inflammation and improved insulin signaling. Endothelial NO levels were also increased as a result of elevated eNOS expression. Conversely, eNOS\textsuperscript{-/-} mice were quick to develop vascular inflammation and insulin resistance regardless of low or high fat diets. In our study, use of the long acting PDE-5 inhibitor could have increased phosphorylated VASP levels as a potential mechanism for ameliorating inflammation, improving insulin resistance and endothelial NO.

We believe that restoration of NO-sGC-PKG signalling following chronic treatment with TAD is the critical mechanism behind improving fasting blood glucose levels and targeting insulin resistance. With improved NO bioavailability and vasodilatation with PDE-5 inhibitors,\textsuperscript{284} there is increased blood flow for muscle glucose utilization and additionally, the decreased TNF-α in the circulation may potentially
attenuate the amount of IRS-1 receptor phosphorylation and improve insulin signalling. It has also been postulated that chronic PDE-5 inhibitor treatment may increase fatty acid oxidation and may also be a potential mechanism for preventing insulin resistance. In a mouse model of diet-induced obesity and insulin resistance, chronic sildenafil treatment was able to improve insulin action and decrease body mass.\textsuperscript{3} In addition, chronic therapy with both a PDE-5 inhibitor and synthetic curcumin, lead to a significant reduction in body weight, which directly correlated with levels of inflammation. We believe the overall reduced inflammatory drive stimulated by adiposity was critical in glucose homeostasis as improvements in fasting glucose correlated with reductions in mean body weight.

Treatment with TAD in C2C12 myoblasts improves oxidative capacity as demonstrated by increase in fatty acid metabolism, including the activities of citrate synthase and 3-OH acylCoA dehydrogenase.\textsuperscript{285} One possible explanation is that PKG has been shown to affect insulin signaling and mitochondrial biogenesis in brown adipose tissue by inhibiting the activity of RhoA and Rho-associated kinase (ROCK), thereby relieving the inhibitory effects of ROCK on IRS-1.\textsuperscript{286} This allows the activation of the phosphotidyl-inositol-3-kinase (PI3K)-Akt signaling cascade downstream of the insulin receptor. In addition, Haas \textit{et al} showed that PKG mediated the ability of NO and cGMP to induce mitochondrial biogenesis and increase the expression of UCP-1, a protein necessary in energy expenditure through thermogenesis.\textsuperscript{286} Similarly, NO and eNOS have been shown to directly correlate with mitochondrial biogenesis as the
abundance of peroxisome proliferator activated receptor-gamma (PPAR-γ) and cold-induced mitochondrial biogenesis were completely abrogated in an eNOS knock-out model.\textsuperscript{287,288}

We found that 12 weeks of treatment with a synthetic analogue of curcumin (HO-3867) provided significant metabolic benefits with improvements in insulin sensitivity and body weight. The anti-inflammatory effects, as demonstrated by reductions in key cytokines and chemokines seen with HO-3867 therapy, were associated with improvements in glycemic control as determined by blood glucose levels, glucose and insulin tolerance tests. Treatment was also associated with a small, but statistically significant, decline in body weight during the 3-month treatment period. Interestingly, a study by Weisberg \textit{et al} also found a notable reduction in hepatic NF-κB activity and an overall decreased inflammatory profile in the \textit{ob/ob} and diet-induced obese murine models after 6 weeks of curcumin therapy. Likewise, they found reductions in body weight and hemoglobin A1c, and improvements in insulin and glucose tolerance tests, and fasting glucose levels. They went on to show a dramatic increase in mRNA and serum protein levels of the insulin-sensitizing protein, adiponectin. In addition, they showed curcumin reduced ER stress and the downstream benefits were improvements in hyperglycemia. They found curcumin therapy upregulated heat shock protein (HSP) 70, HSP90, and Sirt1, which all attenuate the ER stress response and increase adiponectin levels.\textsuperscript{83}
Figure 66. The various potential mechanisms by which curcumin provides anti-inflammatory, anti-oxidant, cardioprotective and insulin sensitizing effects.

This figure illustrates the various pathways by which curcumin has been shown to have protective anti-oxidant, anti-inflammatory, insulin-sensitizing and cardioprotective effects. Curcumin is a cell-permeable compound with inherent histone acetyltransferase (HAT) inhibitory activity for p300/CBP, which attenuates activation of NF-kB. Likewise, through Nrf2 signaling, can upregulate anti-oxidant genes and by inhibition of PKC, will prevent NADPH oxidase from generating ROS. Through the upregulation of AMPK, Akt, PPAR-γ, and CD36 it increases free-fatty acid oxidation and glucose utilization. As discussed earlier, increases in PKG and eNOS also have cardioprotective features. The inhibition of p38 MAPK attenuates cardiac fibrosis and hypertrophy that prevents post-MI induced heart failure, whereas the inhibition of p300/CBP has been shown to be a critical signaling molecule through which curcumin therapy reduces infarct size.
The limitation of the Weinberg study was that an extremely high dose of 1.5g/Kg of curcumin was used because of the compound’s poor oral bioavailability. Such extremely high doses would be impractical to use in human clinical studies, albeit unlikely toxic. However, having similar benefits to organic curcumin but 10 times the absorption rate and potency, HO-3867 may be a promising alternative for future study especially for cardiometabolic diseases. In figure 66, the numerous pathways that curcumin has been shown to provide anti-inflammatory, anti-oxidant and even cardioprotective effects are illustrated. Numerous data supports that curcumin treatment can upregulate glutathione S-transferase and lead to nuclear translocation of Nrf2, which results in an increase in anti-oxidant gene expression.

In addition, through inhibiting the HAT p300/CBP and its transcriptional activating ability, it can block downstream activation of NF-κB, a key regulator of inflammation. Curcumin has also been shown to inhibit specific subunits of NADPH oxidase via PKC, which accordingly attenuates ROS production. Similarly, its affect on metabolism and glucose homeostasis has been shown to be via the upregulation of a number of regulatory signalling molecules such as AMPK, Akt, CD36, PPAR-γ and eNOS.

Like most oral diabetic drugs, curcumin activates PPAR-γ and improves blood glucose levels without having to increase pancreatic β cell secretion of insulin and even induces gene expression of PPAR-γ. Likewise, PPAR-γ is known to be downregulated by TNF-α of which both TAD and HO-3867 decrease circulating plasma levels.
Moreover, curcumin can activate AMPK by phosphorylating its α subunit and increasing fatty acid oxidation within adipocytes. In a study by Seo et al, db/db mice were fed curcumin 0.02% (wt/wt) for 6 weeks. Curcumin significantly lowered hepatic activities of fatty acid synthase, beta-oxidation, 3-hydroxy-2-methylglutaryl coenzyme reductase, lowered plasma FFA, cholesterol, triglyceride concentrations, and increased hepatic glycogen and skeletal muscle lipoprotein lipase (LPL).

Curcumin has been shown to improve insulin signaling and glucose disposal through mitigating oxidative stress within the mitochondria via upregulating the Nrf2 signaling pathway. A recent study by Yu et al showed in an in vivo model that oltipraz, a compound which upregulates Nrf2 signaling, could prevent insulin resistance and obesity in C57BL/6J mice fed a high-fat diet. Within 3T3-L1 adipocytes, curcumin could increase insulin-stimulated glucose uptake through inhibition of NF-κB and JNK, and through inhibition of the pro-inflammatory response by inhibiting phorbol myristate acetate induced (PMA)-induced MCP-1 expression and TNF-α secretion.

Through inhibition of p38 MAPK signalling, SOCS3, and the p300/CBP HAT and via upregulation of the JAK2/STAT3 pathway, curcumin also has favourable effects on cardiac remodeling and cardiac hypertrophy. This abrogates unfavourable remodeling after AMI and reperfusion injury and prevents the development of heart failure.
We found that chronic treatment with TAD had an effect on myocardial expression of miR-103 and miR-107—two miRs that have been shown to play a role in glucose regulation and insulin sensitivity. Chronic TAD therapy attenuated the increase in both miR-103/107 seen in the diabetic controls (DMSO-treated), which was significantly higher than that seen in non-diabetic mice (Figures 60 and 61). Moreover, recent data has shown that the downstream target gene for these miRs include Cav-1, which is a key component of caveolae within the plasma membrane and helps stabilize the insulin receptor and improve insulin signaling.\textsuperscript{219} Given that both miRs negatively regulate Cav-1 expression, \textit{db/db} controls had a significant rise in miR expression and a dramatic decline in Cav-1 expression, which may present one mechanism by which insulin sensitivity is decreased. In contrast, chronic TAD treatment in \textit{db/db} mice lowered myocardial expression of both miRs to levels comparable to that seen in the non-diabetic control. This ultimately led to a significant increase in Cav-1 mRNA transcript levels and protein expression.

The underlying mechanism behind the decreased expression of miR-103 and -107 is still unknown but we speculate it may not be secondary to a single enzyme or signaling molecule but rather a complex system inherently designed to work in concert with the metabolic systems of the body. As hormone dysregulation occurs with over nutrition and obesity, there is upregulation of these miRs, which leads to downregulation of their target gene, Cav-1.
Figure 67. Potential mechanisms by which TAD treatment effects miR-103/107 and Cav-1 expression.

The figure above depicts the possible mechanism through which miR-103 and -107 effect insulin sensitivity and inflammation. Cav-1 provides stabilization of the insulin receptor and enhances insulin signaling, and increased expression of miR-103 and -107 downregulates Cav-1 therefore destabilizing the insulin receptor. Likewise, recent data suggest that this augments inflammatory signaling through TLRs thus increasing inflammation, which further affects insulin signaling. PDE-5 inhibitors increase PKG\(\alpha\) and VASP, and VASP is a protein known to attenuate inflammation. Whether a direct or indirect effect of PKG signaling effects miR-103 and -107 expression has yet to be determined.
Cav-1, being a critical protein within caveolae and known to help stabilize the insulin receptor and enhance insulin signaling, it is only intuitive to realize that proper signaling will be disturbed with its downregulation. Whether treatment with TAD has a direct effect on Cav-1 expression or has an indirect effect as a result of targeting miR-103/107, will take further experimentation most likely with the use of genetically engineered murine models.

In future studies, we will likely include several additional methods to further understand the intricacies of metabolic disease, the NO-cGMP signaling cascade, inflammation and the use of novel pharmacological therapies. The use of nuclear magnetic resonance for body fat composition analysis will be a better method than basic weight measurements to identify fat loss between treatment groups as opposed to simply global weight loss which can include lean muscle mass loss as well. Assessing inflammatory cytokines both pre- and post pharmacological intervention would also be valuable. We would also plan to include a large control set of non-diabetic mice for all experiments. Additionally, given that we did not see a change in IL-6 levels we would pursue the assessment of IL-6 in various tissue samples, especially hepatic and adipose tissue.

Interestingly, a newer device used in human clinical studies called a Dexcom could possibly be used in animal studies. This would dramatically accuracy of blood glucose recordings and furthermore allow for continuous monitoring. The small
implantable device could be implanted under the skin, which would continuously report interstitial glucose levels. The only caveat is that it requires venous glucose samples twice a day to be put into the wireless monitoring device for calibration.

We found that miR-103 and -107 are elevated in the obese, diabetic model and that TAD therapy reduced these levels. Whether a direct correlation to PKG signaling can be made at this time is not possible. In the future, using a PKG viral knockdown model or drug inhibitor and LNA-antagomir therapy/overexpression, whether or not PKG is directly involved or is part of the signaling can begin to be sorted out.

In summary, these studies provide new insight into the potential role of TAD and curcumin-induced cardioprotection, anti-oxidant and anti-inflammatory effects in type 2 diabetes. In particular, we demonstrated that both TAD and HO-3867 attenuated ROS formation induced in the diabetic myocardium following I/R injury and improves mitochondrial integrity while providing cardioprotective effects via Akt-AMPK signaling. In addition, we showed profound effects on insulin sensitivity and glucose homeostasis with both compounds and specifically that TAD may impact the metabolic system through attenuation of key microRNAs (miR-103/107) and their downstream target, Cav-1.

Overall our investigation is unique and may have significant implications for the management and treatment of insulin resistance, oxidative stress and obesity-induced inflammation—three conditions that co-exist long before CV disease clinically manifests.
PDE-5 inhibitors are a promising class of compounds already approved for the treatment of pulmonary arterial hypertension and erectile dysfunction, and curcumin is a natural compound with potent anti-inflammatory, anti-thrombotic, anti-oxidant and cardioprotective effects.\textsuperscript{73, 292} However in its natural form, curcumin is poorly absorbed, has low bioavailability and potency. HO-3867 is a synthetic analogue of curcumin and has been shown to have a 10-fold greater cellular uptake than natural curcumin thus making it an extremely effective, potent and powerful alternative.\textsuperscript{78} Therefore as we move forward, therapeutics designed to target vascular inflammation, improve insulin sensitivity via restoring critical signaling pathways such as the NO-cGMP-PKG pathway and target specific miRs, may prove to be extremely promising in the field of cardiometabolic medicine.
CHAPTER 8

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APPENDICES

Appendix A
Based on the results of the basic science conducted, a translational clinical trial has been initiated using the FDA approved drug TADALAFIL for inflammation, endothelial dysfunction and cardioprotection:

CHRONIC TADALAFIL TREATMENT FOR THE ATTENUATION OF INFLAMMATION AND ENDOTHELIAL DYSFUNCTION IN THE TYPE II DIABETIC PATIENT: A RANDOMIZED DOUBLE-BLIND, PLACEBO-CONTROLLED PILOT STUDY (THE VCU T3 STUDY)

HYPOTHESIS
Insulin resistance and type II diabetes trigger a number of mechanisms and signaling pathways that all lead to tissue injury and functional damage to the cardiovascular system. The exact pathophysiological significance remains to be fully understood, but inflammation and oxidative stress are imperative to the development of vascular
dysfunction. In addition, inflammation plays a central role in vascular disease, from plaque inception to progression and destabilization, which represents a significant paradigm shift from the old idea that vascular disease and atherosclerosis is a problem of lipid accumulation and oxidized cholesterol. This study is unique and may have significant implications for the management and treatment of type II diabetes, insulin resistance and endothelial dysfunction—conditions that exist long before cardiovascular disease clinically manifests itself. Therefore, studying these conditions is extremely relevant since one of our goals is to effectively target and treat the underlying pathology of atherosclerosis in diabetic patients.

a) Type II obese diabetic patients have increased levels of circulating inflammatory cytokines and oxidative stress and as a result of vascular inflammation have endothelial damage and poor nitric oxide availability. This leads to poor blood flow and is at the crux of atherosclerosis.

b) Phosphodiesterase-5 inhibitors have been shown in a number of pre-clinical studies and a few short-term clinical studies to reduce fasting blood sugar values, reduce hemoglobin A1c and improve the amount of microalbuminuria. We propose the chronic daily treatment may also help the diabetic patient improve plasma glucose levels and reduce microalbuminuria through restoring the nitric oxide-cyclic GMP pathway.
SPECIFIC AIMS

Chronic daily treatment for 8 weeks with Tadalafil 5mg for type II diabetic patient will:

- Attenuate cytokines and inflammatory markers (TNF-alpha, IL-1β, IL-6), hs-CRP and homocysteine
- Decrease blood levels of microRNA 103/107 (possible regulators of insulin sensitivity)
- Ameliorate oxidative stress by decreasing levels of 8-isoprostane (a known marker of oxidative stress)
- Increase circulating levels of nitric oxide and urinary cyclic GMP (both indirect measurements of endothelial health)
- Not change urinary cyclic AMP levels thus showing that the primary effects have been mediated through PDE-5 inhibition and not through other PDE inhibition that may result in increased levels of cyclic AMP
- Improve the following commonly followed clinical parameters in diabetic patients:
  - Fructosamine, microalbuminuria and hemoglobin A1c
- Decrease left ventricular volumes (LV end-diastolic and end-systolic volume)
- Reduce pulsed-wave Doppler-derived (PWD) transmitral filling indices (E- and A-wave velocities, E/A ratio) on transthoracic echocardiogram
• Increase left ventricular ejection fraction (LVEF) as measured by the Simpson’s rule, deceleration time [DT], diastolic filling time [DFT], and isovolumetric relaxation time [IVRT] on transthoracic echocardiogram.

• Improve brachial artery flow-mediated dilatation


derived (PWD) transmitral filling indices (E- and A- wave velocities, E/A ratio), increase left ventricular ejection fraction (LVEF) as measured by the Simpson’s rule, deceleration time [DT], diastolic filling time [DFT], and isovolumetric relaxation time [IVRT] on transthoracic echocardiogram.

Tadalafil has been approved by the FDA for the use in erectile dysfunction, pulmonary hypertension (high pressure in the arteries going to the lungs), and an enlarged prostate causing urinary symptoms. In this study, tadalafil will be compared to placebo (a look-alike, inactive substance). Tadalafil has not been approved to lower
inflammation in the body, lower blood glucose levels, or improve heart function—these parameters are being studied in this trial.

The total participation time for each study subject will last up to 12 to 14 weeks. Approximately 40 individuals will participate in this study.

At the first study visit (Visit 1), a comprehensive medical history will be taken and a physical exam will be performed. This exam will include measurements of height, weight and vital signs (pulse, blood pressure and temperature). Blood and urine samples will be collected for routine lab tests. Approximately 2 tablespoons of blood (30mL) will be collected to measure blood levels of inflammation, hemoglobin A1c and nitric oxide. Women of childbearing potential will have a urine pregnancy test done and if positive for pregnancy cannot be enrolled in the study. At the end of the enrollment and procedures (visit 1 and 2), the patient will be randomized by the VCUHS investigational drug pharmacy services and be provided with a 30-day supply of study drug (Tadalafil vs. placebo).

After this initial assessment and blood work, a transthoracic echocardiogram (TTE) will be performed as part of the initial visit of the study (during visit 1), which will be repeated again at end of the study (at 12-14 weeks). This will take place 4 weeks after taking the last dose of the study drug. Various parameters will be recorded from the TTE including: left ventricular volumes (LV end-diastolic and end-systolic volume), pulsed-wave Doppler-derived (PWD) transmitral filling indices (E- and A- wave
velocities, E/A ratio), left ventricular ejection fraction (LVEF) as measured by the Simpson’s rule, deceleration time [DT], diastolic filling time [DFT], and isovolumetric relaxation time [IVRT].

During the same week, the study patient will come for visit 2 after fasting for 6 hours and have a brachial-flow mediated dilatation test. This will measure how well blood is flowing in the artery in the arm which is an accepted marker of endothelial function. This test will be performed at the start of the study and again at the end of the study (at 12-14 weeks). This is a test that measures how well blood is flowing in the brachial artery in using ultrasound to measure flow and dilatation. To perform this test the patient will be asked to come in fasting for at least 6 hours. Lying supine they will have a standard sphygomonanometer cuff applied to the right arm. This will monitor blood pressure and pulse throughout the examination at 5 min intervals. After resting for 5 min, another blood pressure cuff will be applied to your left arm just below the antecubital fossa and inflated 50 mmHg above the measured systolic pressure and stay inflated for 5 min. Baseline images of the left brachial artery will be taken with an linear array multifrequency transducer operating at 9 MHz (GE Logiq 700 devices) just above the cuff for 30 seconds before inflation and then for 2 minutes immediately before deflation to document vasodilator response.

Within 3-4 weeks after Visit 2, the patient will come in for Visit 3. The patient will be asked about his or her health since the last visit and about their overall experience
with the study drug, then have a repeat physical exam, which will include measurements of height, weight and vital signs (pulse, blood pressure and temperature). You will have a small amount of blood drawn (5mL or 1 teaspoon) to check your kidney and liver function as well as fructosamine (to give an estimate of blood sugar values of the past several weeks). If no issues have arisen in the interim, participants will receive a new supply of study drug.

Approximately 7-8 weeks after Visit 2, the patient will come in for Visit 4. The patient will have another physical exam with vital signs documented and again have blood and urine samples collected for testing. Approximately 2 tablespoons of blood (30mL) will be collected to measure blood levels of inflammation, oxidative stress (8-isoprostane), microRNA 103/107, fructosamine and nitric oxide. A urine sample will be collected to measure levels of cGMP, cAMP and microalbumin. Upon completion of a total of 8 weeks of treatment the patient will have completed the drug treatment phase of the study.

Visit 5 will be scheduled at 12-14 weeks from visit 2. At this time a repeat echocardiogram to see if there has been any change in left ventricular ejection fraction and brachial-flow mediated dilatation test to see if there has been any change in flow-mediated dilatation compared with measurements taken from the start of the study. The patient will have the last physical exam and vital signs with blood and urine samples collected for testing. Approximately 2 tablespoons of blood (30mL) will be
collected to measure blood levels of inflammation, oxidative stress (8-isoprostane), microRNA 103/107, hemoglobin A1c and nitric oxide. A urine sample will be collected to measure levels of cGMP, cAMP and microalbumin.

DATA ANALYSIS AND PLAN

The plan is to enroll a total of 40 patients with 20 patients in each arm of the study. The analyses planned include an assessment of blood for markers of inflammation (TNF-alpha, IL-1β, IL-6, hs-CRP), homocysteine, hemoglobin A1c, fructosamine, microRNA 103/107, 8-isoprostane (marker of oxidative stress), urinary cGMP and cAMP, improvement of various parameters on echocardiography and brachial artery flow-mediated dilatation. Standard descriptive statistical analysis will be applied to the data when patient enrollment is completed.

The primary endpoint: Attenuation of the following: inflammatory markers (TNF, IL-1β, IL-6), hs-CRP, homocysteine, microRNA 103/107 levels (possible regulators of insulin sensitivity), 8-isoprostane (marker of oxidative stress); improvement in the following commonly followed clinical parameters for diabetic patients: hemoglobin A1c levels, fructosamine and microalbuminuria. We also propose an improvement in circulating levels of NO and urinary cGMP without significant changes in urinary cAMP.
The secondary endpoint: We propose a decrease in left ventricular volumes (LV end-diastolic and end-systolic volume), reduction in pulsed-wave Doppler-derived (PWD) transmitral filling indices (E- and A- wave velocities, E/A ratio) with increase in left ventricular ejection fraction (LVEF) as measured by the Simpson’s rule, increase in deceleration time [DT], diastolic filling time [DFT], isovolumetric relaxation time [IVRT] on transthoracic echocardiogram. The TTE will be performed and read by same investigator. Furthermore, we propose an improvement in brachial artery flow mediated dilatation from the start to end of the study as a result in improved nitric oxide bioavailability.

Categorical variables (such as tobacco user) will be reported as absolute and percent values. Continuous variables (such as stage of hypertension) will be expressed as median and interquartile ranges. Chi-square and Mann-Whitney tests will be used to compare categorical and continuous variables among the different groups using the SPSS 11.0 Software for Windows.

HUMAN SUBJECTS PARTICIPATION

Participation in the study will require blood samples (approximately 30 mL) at study visit 1, visit 4 and visit 5, and 5mL at visit 3 for a total of 95mL of blood per patient during the entire study period of 12-14 weeks. The blood samples will be analyzed for inflammatory cytokines including TNF-alpha, IL-1β, IL-6, hs-CRP; homocysteine,
hemoglobin A1c, fructosamine, microRNA 103/107, 8-isoprostane, nitric oxide and urine sample will be taken for urine cGMP, cAMP and microalbumin and a urine pregnancy test for women at visit 1 only. A complete metabolic profile will be checked at visit 3, to ensure renal and hepatic function has not changed significantly since the start of the study (one teaspoon or 5mL of blood will be drawn at this visit). Transthoracic echocardiogram and brachial artery flow-mediated dilatation results will be recorded from visits 1, 2, and 5 and will be documented in patient chart, Cerner.

The study will also collect data available in the patient medical record: medical history, prior medications, body weight, vital signs, blood chemistry, hematology, and 12-lead electrocardiogram. If there is no complete metabolic profile and hematology profile available from 60 days prior to visit 1, one will be done prior to start of the study as part of routine care (at visit 1).

INCLUSION AND EXCLUSION CRITERIA

**INCLUSION CRITERIA FOR TADALAFIL TREATMENT FOR TYPE II DIABETICS STUDY (THE VCU T³ STUDY)**

Section 1.01 To be eligible to participate in this study, candidates must have met the following eligibility criteria at the time of enrollment either at the time of enrollment:

1. Age ≥18 or ≤65
2. Currently diagnosed type II Diabetic with current Hemoglobin A1c $\geq 6.5\%$ AND taking one or more oral hypoglycemic agents and/or **stable** insulin therapy (no changes to insulin regimen during 12 weeks of treatment)

3. Known diagnosis of hypertension on at least one anti-hypertensive agent; Stage I or II Hypertension - but $\leq 180/110$ at the time of enrollment

4. Dyslipidemia (must have at least 3 of 4); (LDL$\geq 100$, Total Cholesterol $\geq 200$, Triglycerides $\geq 150$, HDL$\leq 40$ or Non-LDL $\geq 100$) or on medical therapy for dyslipidemia (statin, fibrates, niacin, or ezetimibe)

5. Obesity with BMI $\geq 30$ or waist circumference $\geq 102$cm (40in) for men and 88cm (35in) for women or waist:hip ratio $>0.9$ for men and $>0.85$ for women

**EXCLUSION CRITERIA FOR TADALAFIL TREATMENT FOR TYPE II DIABETICS STUDY (THE VCU T3 STUDY)**

**Section 1.02** Unless otherwise specified, candidates will be excluded from study entry if any of the following exclusion criteria exist at the time of enrollment:

1. Age $<18$ or $>65$

2. Concomitant use of nitrates, alpha blockers, CYP 3A4 inhibitors (including anti-retrovirals for HIV and ketoconazole)
3. Known chronic inflammatory disease (Rheumatoid arthritis, Systemic Lupus Erythematosus, Inflammatory bowel disease, etc.) requiring immunosuppressant therapy including steroids

4. Chronic kidney disease with creatinine clearance ≤ 50 ml/min

5. Liver Disease defined by Childs-Pugh Stage C or D

6. Raynaud's phenomenon

7. Known hereditary retinal degenerative disorders including retinitis pigmentosa

8. Predisposition to priapism such as those with sickle cell anemia, leukemia and multiple myeloma

9. Prior history of priapism

10. Penile deformity (angulation, cavernosal fibrosis, or Peyronie’s disease)

11. Prior myocardial infarction within the past 90 day

12. Unstable angina

13. Uncontrolled hypertension (>180/110)

14. Uncontrolled arrhythmias

15. Systolic dysfunction with left ventricular ejection fraction <40%

16. Stroke within the last 6 months

17. History of seizure disorder

18. Hypotension with blood pressure ≤90/60

19. Patients with New York Heart Association Class III or greater heart failure exacerbation within the last 6 months
20. Patients with left ventricular outflow obstruction, aortic stenosis, idiopathic hypertrophic subaortic stenosis, mitral stenosis
21. Hypersensitivity to phosphodiesterase-5 or phosphodiesterase-3 inhibitors
22. Excessive alcohol intake (≥5 drinks daily)
23. Patients with severe anemia (hemoglobin ≤7 g/dL)
24. Patients with active malignancy or receiving cancer treatment
25. Prisoners
26. Nursing mothers, pregnant women or women planning to become pregnant
27. Prior radical mastectomy on either side (contraindication for brachial artery flow mediated dilatation)
28. Congenital abnormality of either arm or hand (contraindication for brachial artery flow mediated

**RECRUITMENT PLAN**

Patients presenting to the general internal medicine clinic, the cardiovascular medicine clinic or the endocrinology clinic that meet the inclusion criteria will be recruited for this study. Initial contact will be made by one of the co-investigators or the research coordinator who will explain the details of the study and provide the initial information and consent form for patient review. The patient will have up to 3 days to decide after discussing with family, other health professionals, etc. whether or not they wish to participate in the study. If a patient agrees to enroll, contact will be made by the
research coordinator who will ensure inclusion criteria is met and perform an intake evaluation over the phone. She will schedule visit 1 and 2 for the patient at a time when lab-work, echocardiogram and brachial-artery flow mediated dilatation testing (visit 2) can all be conducted and the patient can pick up the investigational drug (after visit 2) from the VCUHS investigational drug pharmacy services for a 4 week supply. The coordinator will also ensure a follow-up appointment within the 3-4 week time period has been made. No vulnerable populations (i.e. children, pregnant women, human fetuses, neonates, prisoners) will be enrolled.

POTENTIAL RISKS

The risks of participation in this study are primarily those resulting from the study drug, Tadalafil. There are some side effects associated with this drug that were documented in phase II and phase III clinical trials where tadalafil was compared to placebo for the treatment of erectile dysfunction or pulmonary hypertension. In clinical trials for once daily use of tadalafil, discontinuation rate was similar to placebo and was less than 1%. The most common side effects (which occurred ≥2%) across all tadalafil trials are:

- Headache
- Flushing
• Heartburn
• Nasal congestion
• Muscle aches
• Back pain
• Limb pain

Rare but dangerous adverse effects that may occur:

• Hypotension: when taken with alpha blockers or nitrates (both of which are contraindicated)
• Priapism (an erection last greater than 4-6 hours that becomes painful)

Extremely rare adverse effects identified in the post-marketing period that were shown to possibly have a causal relationship to tadalafil use include:

• Sudden vision loss in one or both eyes (Non-arteritic anterior ischemic optic neuropathy – NAION)
• Sudden hearing loss or tinnitus

More serious side effects from rare allergic reaction include:

• Stevens-Johnsons Syndrome
• Exfoliative dermatitis
The risk of an allergic reaction such as Stevens-Johnson syndrome or exfoliative dermatitis is extremely rare <1% across all trials conducted. Sudden vision loss, NAION and hearing loss were documented during post-marketing surveillance and tadalafil use and these clinical findings may have had a temporal relationship however it was not possible to determine if a direct relationship existed.

There is some risk with the blood draws that will be taken on 4 visits (visit 1, 3, 4, 5). The total volume of blood is not expected to significantly contribute to overall patient health with 20mL of blood taken at visit 1, 4 and 5 and 5mL taken at visit 3. The transthoracic echocardiogram should not cause any discomfort as the patient will be required to lay comfortably, supine for about 20 min and will have gel placed on the chest wall. An ultrasound probe will be placed on top of the gel to obtain images in various views to visualize the chambers and valves of the heart. There may be discomfort during the brachial artery flow mediated dilatation test during inflation of the blood pressure cuff, which requires the cuff to stay inflated for 5 min. This may cause some pain, sensation of pins/needles or tingling in the arm that will resolve upon deflation of the cuff.
RISK REDUCTION

Throughout the study, several measures will be taken to monitor the safety of the patients. After the patient enrolls in the study, he or she will again be explained all the major adverse side effects and potential life threatening complications. A physician (medically-responsible investigator or a physician co-investigator) will remain on-call and available via pager through the telepage system for research patients at all times throughout the study period. A special virtual pager number for this research study will be created and will be covered at all times. A complete history and physical examination along with comprehensive metabolic panel will be performed at the time of visit one in the event patients will need to be excluded based on most up to date clinical data available. Subjects will be under the observation of VCUHS medical staff during procedures including the transthoracic echocardiogram and brachial artery mediated dilatation study. Furthermore, to minimize potential complications from blood draws such as bruising, pain, irritation, swelling, infection, all blood draws will be performed by skilled nurses from VCUHS.

A steering committee of investigators not involved in the design or execution of the study will be responsible for the monitoring of safety and the evaluation of potential drug-related adverse effects, without breaking the randomization code unless strictly necessary for patient safety. The first interim analysis will be performed after the first 10
patients, and then after half of the patients have been enrolled (20 patients) and at the end of the study (40 patients).

**RISK/BENEFIT**

This study may provide benefit to the patients enrolled given that an improvement in serum inflammatory cytokine and mediator levels (TNF-α, IL-1β, IL-6) and NO, cGMP has been seen with tadalafil use in pre-clinical animal studies. Needless to say, patients randomized to tadalafil may experience no benefit when compared to placebo. The potential benefits of being enrolled include improvement in endothelial function since a dysfunctional endothelium is at the crux of cardiovascular pathophysiology in insulin resistant patients and improvement in endothelial function with chronic PDE-5 inhibitor use has been shown both pre-clinically and in several small clinical studies with sildenafil. We plan to show this by checking urinary levels of cyclic GMP, cAMP, and serum levels of nitric oxide before and after 8 weeks of treatment. Several pilot clinical studies with the use of sildenafil, a shorter-acting PDE-5 inhibitor, showed improvements in endothelial function by measuring urinary cGMP and brachial artery flow-mediated dilatation. The potential knowledge that may come from this study may result in therapies that benefit future type II diabetics and in slowing the progression and development of atherosclerosis along with reducing CV morbidity and mortality in this patient population. The risks associated with the drug are small and
were not significantly different than placebo in the randomized clinical trials conducted with tadalafil for erectile dysfunction and pulmonary hypertension. Furthermore, the more major adverse effects are extremely rare. Nevertheless, we plan to set up a data safety monitoring board and will have a study physician (medically responsible investigator or physician co-investigator) available 24 hours a day/7 days a week throughout the study period.
APPENDIX B

The Effects of Soluble Guanylate Cyclase (BAY 58-2667) in db/db mice after I/R injury

Two separate groups of mice (n=6/group) were treated with 10 mcg/Kg daily for 4 weeks with BAY 58-2667 or an equal volume of 10% DMSO to evaluate the effects of the heme- and NO-independent soluble guanylate cyclase activator on I/R injury. We found a significant reduction of infarct size with BAY 58-2667 treatment (16.6±3.42% vs. 44.7±10.42%, P<0.001) when compared to DMSO.
The Effects of BAY 58-2667 on Circulating Inflammatory Cytokines and Chemokines

Figure 69. The % reduction of circulating cytokines and chemokines after chronic BAY treatment in diabetic mice.

In figure 70, treatment with BAY lead to a dramatic reduction in inflammatory cytokines and chemokines, specifically IL-1β, TNF-α, MIP-1β, and MCP-1. The promising results found with use of BAY 58-2667 in db/db mice should lead to further investigations with this promising compound on its effect on inflammation and various metabolic parameters.
VITA

FULL NAME
Amit Varma

PLACE AND DATE OF BIRTH
Thousand Oaks, California on June 5, 1980

CITIZENSHIP
United States of America

EDUCATION
Fellowship, Cardiovascular Medicine, Division of Cardiology, 2010 – Present
PhD – Department of Physiology and Biophysics, 2008 – 2012
Internship and Residency, Department of Medicine, VCUHS, 2006 – 2008
MD – School of Medicine, Virginia Commonwealth University, 2002 – 2006
BS – Biology with Honors, Virginia Commonwealth University, 1998 – 2002

HONORS/AWARDS/GRANTS
Phi Kappa Phi Honor Society (2012)
Poster Finalist, Mid-Atlantic American College Cardiology Research Competition (2012)
Henry Christian Award, American Federation for Medical Research (2009)
Young Investigator Award, Southern Society for Clinical Investigation (2009)
Cardiovascular Fellowship Grant, American Heart Association (2008)
Finalist, American College of Physicians National Meeting Research Competition (2008)
Winner, American College of Physicians, VA Meeting, Research Competition (2008)
VCUHS Resident/Fellow Research Day Winner (June 2008)
VCUHS Resident/Fellow Research Day Winner (June 2007)
Commonwealth Award in Medicine (2002)
Magna Cum Laude and University Honors graduate (2002)
Phi Beta Kappa Society (1998)
VCU Dean’s List (1998-2002)
PROFESSIONAL MEMBERSHIPS

Medical Society of Virginia (2002 –Present)
American Medical Association (2004- Present)
American Heart Association, Basic Sciences Council (2006 – Present)
American College of Physicians (2006 – Present)
American Physician Scientist Association (2007 – Present)
American Federation for Medical Research (2008 – Present)
American College of Cardiology, Fellow-in-Training (2008 – Present)
Heart Failure Society of America (2012 – Present)
International Society for Heart and Lung Transplantation (2012- Present)

LICENSURES AND CERTIFICATIONS

Diplomate, American Board of Internal Medicine (2009-2019)
Advanced Cardiac Life Support (2006 – Present)
Unrestricted License to practice Medicine, State of Virginia

TEACHING EXPERIENCE

Clinical Instructor, VCU School of Medicine, Foundations of Clinical Medicine Course for MD/PhD, M2 Students (2009-2010)

PUBLICATIONS


**SCIENTIFIC MEETINGS/ABSTRACTS**


15. “Direct Pharmacologic Inhibition of Myeloid Differentiaion Factor-88 as A Novel Therapeutic Target for Acute Myocardial Infarction. BW Van Tassell, FN Salloum, A Varma, NN Hoke, A Abbate. Southern Society for Clinical Investigation, Southern Region-American Federation for Medical Research. February 12th and 13th, 2009, New Orleans, LA. (Awarded SAFMR Young Investigator Award and Travel Award)


23. “Anakinra, Recombinant Human Interleukin-1 Receptor Antagonist Inhibits Apoptosis in Acute Myocardial Infarction” A. Abbate, FN. Salloum, S. Straino S, A.


