New mechanisms in nitric oxide synthase related endothelial dysfunction in the isolated heart

DISSERTATION

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By

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ABSTRACT

Induction of ischemia/reperfusion (IR) injury has been shown to render endothelial nitric oxide synthase (eNOS) dysfunctional; limiting the endogenous mechanisms which regulate vasodilation in the vessel. In the heart, this results in limited tissue perfusion via coronary arteries, which when persistent, results in pump failure. Recently it has been shown that in the ex vivo, isolated heart model, IR results in depletion of the critical NOS cofactor, tetrahydrobiopterin (BH₄). When the lost eNOS cofactor is repleted the activity of the dysfunctional enzyme can be partially ameliorated and vasodilation, while incomplete, is markedly improved. The lack of complete restoration in vasodilation led to this thesis work, which sought to explore the role of reduced nicotinamide adenine dinucleotide phosphate (NADPH), a critical NOS substrate, in enzymatic function after IR injury.

The levels of all pyridine nucleotides where measured throughout ischemia, and subsequent reperfusion to determine any fluctuations in levels as a result of the injurious stimuli. It was found that within the whole-heart, the levels of both NADPH and NADP⁺ (oxidized form of NADPH) were depleted during reperfusion. Furthermore, this depletion appears to be targeted to the endothelium, where the degree of NADP(H) depletion was most severe. Repletion of lost NADPH after IR resulted in a robust increase in coronary flow. When repletion of NADPH was performed with the addition of the eNOS inhibitor,
L-NAME, these benefits were lost. Furthermore, repletion of NADPH was vastly superior to BH₄ repletion, but when given together the improvement to coronary flow was cumulative.

In our model of the isolated heart we show the decline of NADP⁺ coincides with the production of 2′-phospho-cyclic ADP-ribose (2′-P-cADPR), a signaling molecule produced from the ADP-ribosyl cyclase activity of CD38. Originally identified as an antigen marker on B-Cells, CD38 was later found to contain sequence homology with ADP-ribosyl cyclase. It has been widely reported that CD38 activity increases with IR, however these reports only describe this increase in activity as an increase in cADPR, the NAD⁺ analogue, which also has been described as a Ca²⁺ mobilizing agent. Here we show that activity of CD38 from isolated hearts subjected to IR resulted in an increase of 2′-P-cADPR which was 5x that of basal levels. The interaction between CD38 and eNOS was evident when inhibitors to CD38 prevented endothelial loss of NADP(H) and resulted in improved NOS dependent coronary flow over untreated isolated hearts. This link offers a new therapeutic option which could result in improved coronary flow in the face of IR injury.

Finally, the physiological effects of the NADPH-dependent enzyme, glutathione reductase; on eNOS function in the diseased setting of hypertension was explored. Previously, *in vitro* data showed that inhibition of glutathione reductase (GSR) resulted in a new mechanism of eNOS uncoupling, whereby critical cysteine residues of the reductase domain resulted in dysregulation of electron transfer, and subsequent leakage and production of superoxide. Here we show that in aortic rings, this glutathionylation
manifested physiologically as a decrease in endothelial-dependent relaxation. Endothelial dysfunction could be reversed if aorta were incubated with dithiothreitol (DTT), a potent reducing agent, where previously in vitro studies demonstrated DTT could remove glutathionylation. Furthermore, in the hypertensive rat model, it was previously shown that glutathionylation was partially responsible for endothelial dysfunction. Here again we show that treatment with DTT resulted in reversal of endothelial dysfunction and improvement of endothelial-dependent relaxation.

The results from this thesis work show that the role of NADPH is vital in differing disease states within the endothelium, and can manifest as no reflow or hypertension. To improve treatment of these disease states we conclude that NADPH should be targeted as a therapeutic option for treatment.
DEDICATED TO MY MOTHER AND FATHER
ACKNOWLEDGMENTS

First and foremost I would like to thank my parents. My mother and father have made numerous sacrifices to allow me to explore new careers and experiences that I could ever hope for, without them I would not have been able to dream as large as I have. If the words “thank you” were sufficient to show my gratitude I would write it a thousand times, but I don’t think it is, so instead I will continue to strive to be the best person I can be. I will always aspire to be great. I hope you are proud of me.

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VITA

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4. Reyes LA, Biondi R, Varadharaj S, Druhan LJ, Lee HC, Zweier JL. Loss of NADP(H) via CD38 during Ischemia/Reperfusion injury results in a reversible form of eNOS dysfunction in the isolated rat heart. *In Submission* 2012
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2. Cardiovascular Physiology
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<td>AA</td>
<td>Arachadonic Acid</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>AT</td>
<td>Angiotensin receptor</td>
</tr>
<tr>
<td>BH₄</td>
<td>Tetrahydrobiopterin</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<td>cyclic guanosine monophosphate</td>
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<td>COX-1</td>
<td>Cyclooxygenase-1</td>
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<td>CVD</td>
<td>Cardiovascular disease</td>
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<tr>
<td>EDHF</td>
<td>Endothelium derived hyperpolarizing factor</td>
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<tr>
<td>eNOS</td>
<td>Endothelium nitric oxide synthase</td>
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<tr>
<td>EPR</td>
<td>Electron paramagnetic resonance</td>
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<tr>
<td>ET</td>
<td>Endothelin</td>
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<tr>
<td>ETₐ</td>
<td>Endothelin Receptor A</td>
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<td>ECE</td>
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<tr>
<td>GTP</td>
<td>Guanylate cyclase</td>
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<td>$G_s$</td>
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<td>IP$_3$</td>
<td>Inositol triphosphate</td>
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<td>IR</td>
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<tr>
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<td>NADPH</td>
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<td>NO</td>
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<td>NOHA</td>
<td>N-hydroxyl-L-arginine</td>
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<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
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<td>O$_2$</td>
<td>Oxygen</td>
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<td>ONOO$^-$</td>
<td>Peroxynitrite</td>
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<td>PGI$_2$</td>
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<td>Prostacyclin synthase</td>
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<tr>
<td>IP</td>
<td>Prostacyclin Receptor</td>
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<tr>
<td>K$^+$</td>
<td>Potassium</td>
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</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKG</td>
<td>Protein kinase G</td>
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<tr>
<td>RAS</td>
<td>Renin-angiotensin system</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
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<td>SR</td>
<td>Sarcoplasmic reticulum</td>
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<td>WT</td>
<td>Wild-Type</td>
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<td>XO</td>
<td>Xanthine oxidoreductase</td>
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CHAPTER 1
INTRODUCTION

1.1 General Introduction

With the exception of the 1918 influenza epidemic, since the year 1900 cardiovascular disease has been the leading cause of mortality in the United States [1]. In 2008 (the latest year in which data is available) nearly 2400 Americans died daily from cardiovascular complications or one every 37 seconds [1]. In the same year (2008) more people died of cardiovascular disease (CVD) than cancer (all forms), chronic lower respiratory disease, diabetes mellitus and accidents combined [1].

Cardiovascular disease is often thought of as an age associated disease. Indeed, as one ages the risk for developing cardiovascular disease increases (fig 1) however, approximately 20% of all cardiovascular related deaths in 2008 were under the age of 65 [1]. Notably, the number of deaths in the United States from cancer (all forms) and CVD is nearly identical across all age groups, with an increasing dichotomy in older age groups (fig 1) [1]. This is true for both sexes, and amongst all racial and ethnic classes, consistently the number of mortalities due to cardiovascular disease is considerably higher than deaths from cancer (all forms) (fig 2) [1].
The impact of CVD extends beyond mortality, as the burden of treatment economically is profound, where 1 out of every 6 dollars spent on healthcare is related to CVD treatment. This translates as an annual cost to Americans of nearly 150 billion dollars each year [2]. When CVD is the principle diagnosis the average length of hospital stay is approximately 4.6 days, costing nearly $23,000 per admission. Furthermore, $11.7 billion was paid to Medicare beneficiaries for in-hospital costs when CVD was the principle diagnosis [2].

While CVD is the leading cause of death for Americans, the advancements in intervention and treatment of critical events have improved dramatically. From the year 1998 to 2008 (the latest year data was made available) deaths from CVD have declined 14.1% [1]. In order to improve upon these numbers it is vital to continue to advance and explore new therapeutic interventions in the search for the elimination of CVD.

Dysfunction in the mechanisms which regulate patency often precede CVD states such as myocardial infarction (MI) (fig 3), of which 1.5 million occur every year in the US [1]. The regulation of blood flow through the patent vessel is central to this thesis work and is discussed further below.

1.2 Blood flow through systemic circulation

The blood flow through the systemic circulation can be described in part by Poiseuille’s law:

\[ \dot{Q} = \frac{\Delta P \pi r^4}{8\eta L} \]
Where \( \dot{O} \) is blood flow; the parameters which govern its movement through a rigid tube are the pressure difference across the tube (\( \Delta P \)), the radius of the tube (\( r \)), the viscosity of the tube (\( \eta \)) and its length (\( L \)). Again, Poiseuille’s law only partly describes blood flow through the vessels since the properties of the rigid tube and blood vessels are different. In particular the elastic and branching nature of the blood vessels (fig 4) add to complexities which are not described in Poiseuille’s law, however even with its limitations blood flow through the systemic vasculature can be estimated using Poiseuille’s law.

As mentioned previously the pressure difference across the vessel plays an important role in blood flow. In the large arteries such as the aorta the pressure across the vessel can be as high as 120 mmHg in systole. As the aorta gives way to the smaller arteries (e.g. carotid artery) the pressure is lowered to approximately 110 mmHg in systole. This pressure difference helps blood flow move continuously throughout the body as pressure drops continuously to nearly 0 in the great veins (superior/inferior vena cava).

Blood flow is inversely proportional to the viscosity of blood and the length of the vessel. Where the length of the systemic vasculature is generally constant, the blood viscosity can change. For example during periods of dehydration, the blood hematocrit (combination of red blood cells and plasma) favors red blood cells, which is more viscous.

Finally, like the pressure gradient, blood flow is proportional to the radius of the blood vessel. The importance of the radius is highlighted mathematically by its exponent.
Indeed in the systemic circulation, this becomes the most important regulator for blood flow. The regulation of vascular tone is controlled predominately by the endothelium which line the vessels, the mechanisms by which these cells regulate tone is of critical importance to findings of this work and is discussed in depth below.

1.3 Coronary Endothelium and the regulation of vascular relaxation

The system of vessels which supply the heart muscle is termed the coronary arteries, and when they fail to supply blood to the heart muscle this can lead to a MI and cause irreparable damage, which when left untreated can cause pump failure and death. Within the coronary arteries vasodilation is primarily regulated by nitric oxide (NO), prostacyclins and endothelial derived hyperpolarizing factor (EDHF). Along with chemical stimulation, mechanical stimulation can also cause vasodilation via a change in sheer stress, which is termed flow-mediated vasodilation. These various factors which regulate vascular function will be reviewed in the subsequent sections.

1.3.1 Nitric Oxide synthesis and regulation of vascular tone

In the coronary vasculature NO is primarily synthesized via the enzyme endothelial nitric oxide synthase (eNOS) [3-5]. eNOS is a 280 kDa calmodulin (CaM) dependent protein comprised of a reductase and oxygenase domain [6, 7]. Under basal conditions eNOS is bound to Caveolin-1 rendering the enzyme inactive [8, 9]. However, an increase in endothelial intracellular calcium (Ca^{2+}) from endogenous substances (eg bradykinin, serotonin, histamine etc.) or pharmacological agents (substance P and
acetylcholine) results in the activation of CaM [8]. Once activated CaM will bind to eNOS, which results in eNOS disassociation from Caveolin-1 and subsequent activation [10]. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) undergoes a hydride transfer with flavin adenine dinucleotide (FAD) of the reductase domain, which then donates an electron to flavin mononucleotide (FMN). The accepted electron is passed from FMN to the heme of the oxygenase domain; reducing it from the ferric to the ferrous state. In its ferrous state the heme is now free to bind molecular oxygen (O₂); forming the superoxy-heme intermediate [11, 12]. Of critical importance is the eNOS cofactor, BH₄, which quickly donates a second electron and stabilizes the intermediate and forms a heme-iron (IV) oxo-species [11, 13]. The heme-iron (IV) oxo-species reacts with L-Arginine (L-Arg) to form the intermediate N-hydroxy-L-arginine (NOHA) [11]. Another electron passes through the reductase domain and forms another heme-iron (IV) oxo-species which reacts with the newly formed NOHA, resulting in the release of NO and the byproduct L-citrulline (L-Cit) (Fig 5) [11, 13].

Since NO is a gas it is free to diffuse out of the endothelium and into the smooth muscle where it binds to the heme moiety in soluble guanylate cyclase (sGC) and forms a nitrosyl-heme adduct, which displaces the iron in the porphyrin ring. This displacement allows guanosine triphosphate (GTP) to bind and be hydrolyzed, leading to the formulation of cyclic guanosine monophosphate (cGMP) [14]. cGMP binds protein kinase G (PKG), which phosphorylates numerous proteins leading to its effects on smooth muscle relaxation [14]. In particular, PKG activates myosin light chain phosphatase (MLCP), which in turn dephosphorylates myosin light chain, the regulatory
subunit for contraction in smooth muscle cells (SMC); this leads to a decrease in sensitivity to Ca\(^{2+}\) and results in relaxation [15, 16].

While eNOS is of central importance in my work; as explained previously, it is only one of a number of molecules which are relevant in endothelial dependent regulation of relaxation. Therefore, these other molecules will be discussed in brief in the following sections.

1.3.2 Prostacyclin and vasodilation

Cyclooxygenase 1 (COX-1) is an enzyme constitutively expressed in a wide variety of tissues including the coronary vasculature. COX-1 is a bifunctional enzyme which acts as an endoperoxidase synthase on arachadonic acid (AA) by oxygenating and cyclizing AA into endoperoxidase prostaglandin G\(_2\) (PGG\(_2\)) [17, 18]. The enzyme then acts as a peroxidase and converts PGG\(_2\) into PGH\(_2\), which can be converted into a wide range of molecules depending on the synthase used. When PGH\(_2\) is in the presence of prostacyclin synthase (PGIS) it is converted into the vasodilator prostacyclin (PGI\(_2\)) [19]. Prostacyclin mediates vasodilation through binding of the prostaglandin I\(_2\) receptor (IP), which is a G-protein coupled receptor. Once PGI\(_2\) is bound, the IP receptor undergoes a conformational change, releasing the G\(_s\) subunit which in turn activates adenylyl cyclase (AC) [20, 21]. The active AC converts adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP), the second messenger responsible for activation of protein kinase A (PKA) [21, 22]. PKA phosphorylates myosin light chain kinase (MLCK), decreasing its activity [23]. With MLCK rendered inactive the myosin light
chain (MLC) remains in the unphosphorylated state, decreasing its sensitivity to Ca$^{2+}$ within the SMC and promotes relaxation over constriction [23].

**1.3.3 Endothelial derived hyperpolarizing factor**

While NO and PGI$_2$ account for a large part of agonist mediated increases in relaxation, they do not account for it entirely. The discovery of endothelium derived hyperpolarizing factor (EDHF) helped explain the portion of relaxation that is NO and PGI$_2$ independent. [24-26]. The mechanism of action for EDHF is complex and has not been fully elucidated; however, it is apparent that EDHF works in multiple steps. First, ligand induced binding on the endothelial cell results in increased Ca$^{2+}$ via both intracellular and extracellular stores [24-26]. The increased Ca$^{2+}$ results in both the activation of Ca$^{2+}$ sensitive potassium (K$^+$) channels and the synthesis of EDHF [27, 28]. Activated K$^+$ channels in the endothelium results in K$^+$ efflux, and subsequent hyperpolarization of the endothelial cell [28]. Newly synthesized EDHF moves from the endothelial cell to the SMC and causes activation of K$^+$ channels [29-32]. Along with sensitization of K$^+$ channels, EDHF has been shown to inhibit Ca$^{2+}$ channels of the SMC. The efflux of K$^+$ through the EDHF sensitive K$^+$ channel and the inhibition of Ca$^{2+}$ movement results in SMC hyperpolarization and relaxation [30].

The actual molecule relating to EDHF is under intense debate, but it is widely thought to be either hydrogen peroxide (H$_2$O$_2$), c-type natriuretic peptide, a byproduct from cytochrome p450 or possibly a combination of these molecules [31]. It is important to note that throughout the systemic vasculature (including coronary vasculature) the
regulation of vasodilation appears to be mediated by different molecules. It has been shown that in larger vessels (e.g. aorta) NO and PGI\textsubscript{2} are predominately responsible for vasodilation, where in the microvasculature (i.e. arterioles) EDHF is the primary regulator [33, 34]. Finally it has been argued that EDHF can act as a secondary mechanism when NO can no longer function in vasodilation, offering redundancy in vascular regulation of vasodilation [35].

1.3.4 Flow mediated vasodilation

The flow of blood through vessels can also mediate changes in vascular diameter resulting in vasodilation [36, 37]. An example of this occurs during exercise, when increased blood flow to skeletal muscle increases sheer stress on the vascular walls [38]. The increased sheer stress results in a CaM mediated increase in eNOS activation, via increased intracellular Ca\textsuperscript{2+} within the endothelium, much like what occurs during chemical stimulation (i.e. bradykinin, acetylcholine or histamine) [39]. Subsequent NO formation leads to GC activation resulting in cGMP production, where the downstream effect leads to SMC relaxation [39]. However, unlike substrate binding (e.g. histamine); during increased sheer stress c-Src, a tyrosine kinase, is activated and increases transcription of eNOS as well as the stabilization of eNOS mRNA, thus sheer stress also has a long term action as well as the transient action of vasodilation [40].

1.4 Coronary Endothelium and the regulation of vascular constriction
While the endothelium is critical in the maintenance of vasodilation through the aforementioned processes, it also is critical in the regulation of vasoconstriction. The molecules preproendothelin and PGH₂ can be cleaved by endothelial enzymes into their active forms where they can function as vasoconstrictors [41, 42]. Angiotensin II (Ang II) is a molecule whose precursor is secreted by the kidney, but its action can influence the coronary vasculature through its vasoconstrictive effects [43, 44]. Vascular homeostasis is a critical function that is largely dependent on endothelial function. In the setting of a variety of diseases such as ischemia/reperfusion injury (discussed in 1.5.1) the endothelium can be rendered dysfunctional; the result of which can be hyper-constriction or hyper-relaxation, both of which can be life-threatening. Therefore, the maintenance of endothelial integrity and its ability to maintain both constriction and dilatation is extremely important. Is it was discussed above (section 1.3) the primary mechanisms for endothelial-dependent vasodilation, therefore the vasoconstrictive properties of the endothelium will be considered below.

1.4.1 Role of endothelin, thromboxane and Angiotensin II in vasoconstriction

Within the endothelium the endothelin (ET) precursor prepro-ET is cleaved into big-ET by the furin-like endopeptidase; which can then be converted to the active ET₁ via ET-converting enzyme (ECE) [41, 45]. The active form, ET₁, can then act on the surrounding environment in a paracrine or autocrine fashion by binding to the ETₐ or ETₐ receptor [46, 47]. The receptors are in large part the modulator of downstream function of ET; when ET binds ETₐ, a G-protein coupled receptor located on SMC, the result is
activation of phospholipase C, which liberates intracellular calcium through an inositol triphosphate (IP$_3$) dependent mechanism; leading to vasoconstriction [48, 49]. However, when ET binds ET$_B$ located on the endothelium the resulting action is the release of NO and PGI$_2$ (both vasodilators) as well as the inhibition of ECE. In addition to NO and PGI$_2$ the binding of ET$_B$ leads to activation of its G$_a$ subunit leading to inhibition of cAMP formation and an increase in phosphoinositide hydrolysis, thus limiting intracellular Ca$^{2+}$ and promoting relaxation [50-52].

It was discussed in section 1.3.2 that PGH$_2$ was the precursor to the vasodilator, PGI$_2$. However, PGH$_2$ can also be converted within the endothelium to thromboxane A$_2$ (TxA$_2$) by thromboxane synthase [42]. TxA$_2$ acts on the SMC via a G-protein coupled receptor mechanism [53, 54]. Activation of the thromboxane A$_2$ receptor (TP) results in increased Ca$^{2+}$ via phospholipase C (PLC) release of IP$_3$; in turn this results in Ca$^{2+}$ release from the sarcoplasmic reticulum (SR). The downstream effects of the increased Ca$^{2+}$ are phosphorylation of the MLC of smooth muscle increasing sensitivity to Ca$^{2+}$ and constriction [42, 55].

Ang II is a product of the renin-angiotensin system (RAS) which binds to the angiotensin II smooth muscle receptor termed AT$_1$ [56]. When Ang II binds to AT$_1$, a G-protein coupled receptor, this results in a conformational change in the receptor and release of the G$_q$ subunit [57]. This results in the liberation of diacylglyceride (DAG) and IP$_3$, where IP$_3$ release leads to increased Ca$^{2+}$ from intracellular stores, and DAG results in phosphorylation of PKC, the downstream action of both is SMC contraction [58-60]. In addition to Ang II effects on vasoactivity it is noteworthy that it also plays a role in the
generation of free radicals, especially in the disease setting such as hypertension. As will be discussed later (section 1.5.1.2), increased production of superoxide can overwhelm and inactivate eNOS; resulting in the cessation of NO production [61-65]. In addition to NO cessation superoxide can non-specifically activate phosphatases, thus while IP₃ and DAG mediate the primary pathway of action, induction of superoxide by Ang II can prolong vascular constriction through eNOS inactivation and dephosphorylation of MLC [66, 67].

1.5 Cardiovascular Disease

It was discussed in section 1.1 the medical and economic burden of CVD; of which coronary artery disease (CAD) is a prevalent form. CAD begins as endothelial dysfunction, which disrupts vascular homeostasis and limits vasodilatory function through impairment of the aforementioned processes (sections 1.3 & 1.4). In particular, loss of nitric oxide leads to an increased inflammatory response; where macrophages infiltrate vascular walls and form atherosclerotic plaques. Rupture of the atherosclerotic plaque leads to thrombus overlay and complete (or near complete) occlusion of the artery, this event is termed acute coronary syndrome (ACS), and if not treated immediately can lead to a myocardial infarction. Of primary concern for this thesis work is the treatment for MI, which leads to the new problem of ischemia/reperfusion injury and is described below.

1.5.1 Ischemia/reperfusion injury
Treatment and alleviation of coronary occlusion currently is achieved by thrombolytic therapy, percutaneous, or surgical intervention; the success of which relies heavily on quickly reintroducing blood flow to the once ischemic tissue [68]. While restoration of blood flow is critical, and necessary to prevent cardiac myocytes death [69, 70] it introduces an additional problem of reperfusion injury [65, 71-73]. The phenomenon of ischemia/reperfusion (IR) injury leads to (among other problems) endothelial dysfunction, which results in decreased vascular relaxation, and increased vascular permeability leading to edema [74]. The combination of increased vascular permeability and decreased vasorelaxation contribute to the limitation in reperfusion therapy of the ischemic tissue [74]. When limited blood flow through the coronary arteries persists despite reperfusion to the once ischemic tissue it can slowly lead to cardiac myocyte death, and pump failure [75].

The phenomenon of IR injury was originally described by Jennings and colleagues [76]. At the molecular level IR injury predominantly affects two cell types in the heart, myocytes and endothelium, in different but equally detrimental manners.

1.5.1.1 Ischemia/reperfusion injury effects on cardiomyocytes

In Jennings’ seminal findings he notes that in dogs undergoing 30 minutes of coronary ligation followed by reperfusion there is a significant change in myocyte ultrastructural appearance. In the area of infarct; there was a lack of uniformity in the necrosis of myocytes with their organelles becoming extremely disorganized [76]. The group of dogs which received permanent ligation displayed a much different
ultrastructural appearance; where the area of necrosis was much more organized and only cells in the peri-infarct region showed the disorganization that ligation with reperfusion caused [76]. Subsequent characterization of ultrastructural changes from others noted swollen mitochondria with disrupted cristae, hypercontracted sarcomeres, depleted glycogen stores and nuclear damage [77, 78]. The change in ultrastructure has since been elucidated through a number of studies. The cessation of oxygen and nutrients to the tissue results in an abrupt stop to oxidative phosphorylation [79]. As the myocyte attempts to maintain ATP production through glycolysis the intracellular pH begins to decline as lactate increases [79]. The ATP produced from glycolysis is not sufficient to maintain contractile function, nor the ion pumps which maintain the electrochemical membrane gradient [80]. The loss of the electrochemical gradient leads to Ca$^{2+}$ influx, which causes a widespread and nonselective activation of a number of kinases, further exacerbating the bioavailability of ATP [81, 82].

With the onset of reperfusion, oxygen and nutrients are rapidly restored, however the re-introduction of oxygen to the disrupted electron transport chain results in free radical ($O_2^-$) production from NADH dehydrogenase [61, 83, 84]. This increase in free radicals leads to non-reversible protein modifications, and results in a loss of membrane integrity within the mitochondria [83, 85]. Leakage of cytochrome C, a mitochondrial specific protein, via the mitochondrial transient transition pore (MTTP) activates caspases and leads to cell death [85].

Historically, the primary focus has been on rescuing cardiac myocytes; however emphasis has also shifted to the endothelium of coronary arteries [63, 65, 86, 87].
1.5.1.2 Ischemia/reperfusion injury effects on coronary endothelium

At the molecular level the reintroduction of blood flow causes a sudden burst of free radicals within the coronary endothelium, [88, 89] which converts tetrahydrobiopterin (BH$_4$) to dihydrobiopterin (BH$_2$) [90-92]. Of note, it has also been demonstrated that BH$_4$ may also be converted to dihydroxanthopterin (XPH$_2$) through non-enzymatic side-chain cleavage [62] (fig 6). Electron paramagnetic resonance (EPR) spectroscopy studies have shown that BH$_4$ stabilizes the ferrous-dioxygen complex of the oxygenase domain of eNOS by quickly donating an electron to the complex, allowing L-Arg oxidation [91-93]. In the absence of BH$_4$ the enzyme is uncoupled and the ferrous-dioxide complex dissociates, releasing superoxide (O$_2^-$) [11]. Uncoupled eNOS is defined as a state in which the enzyme is converted from a NO synthase to a O$_2^-$synthase (Fig 7). During reperfusion the endothelium posses a mixture of coupled and uncoupled NOS, resulting in the production of both O$_2^-$ and NO, which together react and form peroxynitrite (ONOO$^-$). ONOO$^-$ is a powerful oxidant and nitrating agent capable of damaging a spectrum of bioactive molecules [94].

Physiologically this is manifests as endothelial dysfunction, and as a result the coronary vasculature has a limited ability to vasodilate, and becomes a pro-thrombotic/pro-inflammatory environment [95, 96]. These events can lead to re-oclusion and the no reflow phenomenon, limiting therapeutic intervention [96-98]. The goal of this work seeks to expand beyond the work with the eNOS cofactor BH$_4$ into other possible
enzyme substrates which may become limiting in the setting of IR injury and blunting endothelial recovery.

1.6 NADPH as a critical substrate

As discussed in section 1.5.1.2 the essential nature of BH₄ for proper eNOS function is clear. However, in the setting of IR injury the eNOS substrate NADPH is of equal importance. NADPH is a substrate for many enzymes involved in IR injury, where NADPH is used as a reducing agent to re-synthesize glutathione as well as BH₄ (fig 8). The processes by which NADPH is involved in the formation of the antioxidant and eNOS cofactor are discussed in depth below.

1.6.1 Role of NADPH in glutathione synthesis

In normal cells, free radical generation is kept in balance by a number of enzymes including glutathione peroxidase (fig 9). Thus far there have been several mammalian glutathione peroxidases (Gpx) discovered, each differentiated by its location within specific tissues and cellular compartments [99]. Of particular interest, Gpx1 is found intracellularly in most mammalian tissues, and acts in redox balance [99, 100]. Gpx1 (as well as other Gpx isoforms) binds oxidants, such as hydrogen peroxide (H₂O₂) by catalyzing the following reaction [100]:

\[
2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\text{H}_2\text{O}
\]
The reactive site of Gpx1 contains a selenium ion which binds peroxide, once bound the antioxidant, glutathione (GSH), can react and form the Se-GS complex [101]. The reactive site is restored, once another GSH molecule binds, breaking the Se-GS bond and forming free glutathione disulfide (GSSG) and H₂O [101].

The function of GSH as an antioxidant in cellular detoxification is predicated on not only the activity of GPx but also the recycling of GSH from GSSG, thereby allowing further reactions with oxidants. Recycling of GSSG back to GSH is accomplished by the enzyme glutathione reductase (GSR) and its substrate NADPH [102, 103]. GSR restores GSH levels via the following reaction:

\[ \text{GSSG} + \text{NADPH} \rightarrow 2\text{GSH} + \text{NADP}^+ \]

The importance of GSR and eNOS function was highlighted in the work by Chen et. al., where 1-3-bis(2-chloroethyl)-1-nitrosourea (BCNU), an inhibitor of GSR, resulted in increased GSSG levels, which when not converted back to GSH resulted in glutathionylation of important cysteine residues on eNOS. Glutathionylation of eNOS lead to uncoupling and free radical generation, which was different from BH₄ dependent uncoupling [104]. Thus, not only is the function of GSR important in the process of antioxidants, but also in eNOS function. The physiological effects of this new mechanism of eNOS uncoupling is further discussed and explored in chapter four.

1.6.2 Dihydrofolate reductase and NADPH
Within the endothelium, there exist two enzymes which act to quickly restore BH₄ levels when it is converted to quinoid-dihydrobiopterin (qBH₂) or BH₂ [92, 93, 105]. Neither qBH₂ nor BH₂ have the capacity to donate an electron and stabilize the heme-superoxy intermediate of eNOS, leading to superoxide generation [92, 93]. Dihydropteridine reductase (DHPR) uses reduced nicotinamide adenine dinucleotide (NADH) to convert qBH₂ back to BH₄ [106, 107], while dihydrofolate reductase uses NADPH as its reducing equivalent to convert BH₂ back to BH₄ (fig 6). Crabtree et. al., elegantly showed a link between DHFR function and eNOS function. Through in vitro studies in cell lines, knockdown of DHFR via siRNA or inhibition with methotrexate resulted in decreased conversion of BH₂ to BH₄ essentially reducing intracellular levels of BH₄ [90]. The reduction in BH₄ levels was enough to induce uncoupling of eNOS and production of O₂⁻[90].

It is clear from the above sections that NADPH plays a critical role in direct eNOS activation and in the maintenance of critical eNOS cofactors and/or residues which allow proper function. Therefore it is logical that any disruption in NADPH bioavailability would be detrimental to vascular function.

1.7 Specific Aims, Objectives, and Rationales

The following specific aims and objectives have been developed in an attempt to elucidate the role NADPH plays in maintaining proper eNOS function in different disease states (e.g. IR injury and hypertension) which have been demonstrated to induce endothelial dysfunction. The answers from these aims will illustrate NADPH to be a
novel therapeutic target for the restoration of NOS function in pathological states. With these aims a hypothesis was developed and conclusions from them were drawn.

1.7.1 Determine the modulation IR injury has on the eNOS cofactor NADPH and its physiological significance.

Nicotinamide adenine dinucleotide phosphate (NADPH) is a critical substrate for the transfer of electrons to the reductase domain of eNOS. Without it, the enzyme cannot form its product, NO. It has not been shown whether NADPH, like BH₄, can become limiting post-ischemia. Therefore, to address this aim the technique of Langendorf was used to isolate hearts. Isolated hearts were subjected to varying levels of ischemic and reperfusion injury. After which, the levels of NADPH was determined from these hearts using high performance liquid chromatography (HPLC). To determine if modulated levels of NADPH in IR injury are physiologically relevant, Langendorff-perfused isolated hearts were again used and subjected to IR injury. However, in these studies NADPH was infused using a liposomal formulation after the onset of IR injury. The benefits of this delivery where compared to other isolated hearts which received either BH₄ alone, NADPH + BH₄ or NADPH + L-NAME. The results from these experiments showed NADPH delivery to isolated rat hearts undergoing IR injury was not only physiologically relevant, but also clinically applicable. The efficaciousness of NADPH therapy was determined through its comparative results in improving eNOS dysfunction with respect to BH₄ repletion therapy.
1.6.2 Determine the cause of ischemia/reperfusion injury induced alterations in NADPH levels and if this process can be ameliorated.

While oxidative stress is a profound marker of IR injury and may contribute to altered levels of NADPH via oxidation to NADP⁺; it is possible that these levels are modified due to a more complex mechanism involving modifications to the production/consumption of NADPH. Therefore, in this aim both the production and consumption of NADPH were explored during ischemia/reperfusion injury to determine effects on NADPH levels. Glucose-6-phosphate dehydrogenase (G6PD) is predominately responsible for reducing NADP⁺ to NADPH, thus maintaining levels of the reduced form. In this aim we determined if altered enzymatic function of G6PD could contribute to depleted NADPH levels during IR injury. It may prove that the capacity of re-synthesis of NADPH from NADP⁺ is intact, thus the mechanism for altered levels of NADPH may be linked to overconsumption; one possibility is linked to the activation of CD38, an enzyme which has been shown to utilize NADP⁺ as its substrate. NADP⁺ is the oxidized form of NADPH; accordingly any alteration in these levels could affect the intracellular levels of NADPH. Therefore it was determined whether CD38 activity increased a result of IR injury. Any fluctuation in CD38 levels may limit NADP⁺ bioavailability for NADPH synthesis and in turn limit the availability of the eNOS substrate in NO synthesis. If CD38 is indeed functioning in this capacity then any inhibition of NADP⁺ consumption through CD38 metabolism should restore NADPH bioavailability (with a functioning G6PD) for eNOS consumption thus ameliorating endothelial dysfunction as a result of IR injury.
1.7.3 *Determine significance for inhibition of the NADPH-dependent enzyme, Glutathione Reductase, in antioxidant synthesis.*

Recently it was discovered that a new mechanism for eNOS uncoupling involved glutathionylation of critical cysteine residues in the reductase domain. This aim explored the physiological significance of glutathionylated eNOS, whose mechanism involves inhibition of the NADPH-dependent glutathione reductase (GSR). This will be accomplished using the *ex vivo* prep of aortic rings, which will be subjected to incubation with the GSR inhibitor, 1-3-bis(2-chloroethyl)-1-nitrosourea (BCNU). If the *ex vivo* studies follow the *in vitro* studies of Chen et al.; then acetylcholine stimulated vasodilation from the aortic rings will be blunted, as a result of the uncoupled eNOS.

Also, in this aim, the clinical significance of glutathionylated eNOS was explored. Chen et al. found that in spontaneous hypertensive rats (SHR), eNOS derived from aorta was glutathionylated. Again, *ex vivo* preps of aortic rings were used, and the possibility of eNOS restoration via the removal of glutathionylation was determined. This was accomplished by incubating the aortic rings with the powerful reducing agent dithiothreitol (DTT). The practicality of reversing eNOS glutathionylation as a therapeutic approach for hypertension was accomplished by measuring blood pressure of hypertensive rats who receive IP injections of reducing agents.

1.7.4 *Overall Goal & Significance.*
The overall goal of this study is to elucidate the mechanisms involved in endothelial dysfunction as it relates to disease states such as, IR injury and hypertension. While this problem is multifaceted one major factor leading to endothelial dysfunction is eNOS dysfunction and the inability or reduced ability to produce NO in the post MI and hypertensive coronary arteries. While much literature exists on the role the critical cofactor BH$_4$ plays in eNOS function in these disease states, very little exists on NADPH and how it is affected and if any modulation would result in decreased NO production. The findings described in this thesis will illustrate whether depletion of NADPH, or modulation of the NADPH-dependent enzyme GSR, could be limiting in the restoration of eNOS function in IR injury or hypertension. The aforementioned aims were designed to highlight possible therapeutic approaches to ameliorate NADPH depletion and improve endothelial dysfunction.
Figure 1 - Cardiovascular disease (CVD) mortality vs. cancer mortality rates. The rates for CVD and cancer mortality is nearly identical in age groups <74 years of age, however a large divergence begins in the subsequent age group and continues in those >85 years of age. Total mortality from CVD is nearly 30% higher than from cancer (all forms). Figure is from The American Heart Association’s report: *Heart disease and Stroke Statistics – 2012 update.*
Figure 2 - Mortality from cardiovascular disease (CVD) separated by sex. Total mortality rates from CVD varies between sexes, however in both men and women, the leading cause of death is still CVD, which is considerably greater than cancer, accidents, chronic lower respiratory disease (CLRD), diabetes and Alzheimer’s disease. Figure is from The American Heart Association’s report: *Heart disease and Stroke Statistics – 2012 update*. 
Figure 3 - Vascular endothelial dysfunction (VED) in cardiovascular disease. VED is often the first step in the progression of many different cardiovascular diseases (i.e. hypertension, atherosclerosis and thrombus formation), however VED can also be an end result of certain risk factors/disorders.
Figure 4 – Blood flow through the systemic circulation. Blood is actively pumped out to through the aorta, which then gives way to large arteries. As the diameter of the vessels decrease the vascular resistance increases, where the small arteries, arterioles and capillaries account for nearly 90% of vascular resistance. Gas and nutrient exchange occurs in the capillary beds of tissues. Blood is returned via the venous system which ends with the vena cava returning blood to the right atrium. Figure adapted from Medical Physiology; by RA. Rhodes & GA. Tanner.
Figure 5 - Normal function of eNOS. NADPH is the first electron donor of the reductase domain. Electrons pass from the reductase domain to the oxygenase domain where the heme is reduced from the ferric to the ferrous state, and forms the superoxy-heme intermediate once \( \text{O}_2 \) is bound. \( \text{BH}_4 \) donates an electron thereby stabilizing the perferril-intermediate and results in the formation of either NOHA or NO, depending on the substrate.
Figure 6 - Tetrahydrobiopterin (BH$_4$) and its breakdown products. BH$_4$ is a critical cofactor for eNOS derived NO synthesis. Oxidation of BH$_4$ is reversed via two enzymes, dihydropteridine reductase or dihydrofolate reductase, the latter of which is NADPH dependent. Non-enzymatic side chain cleavage can also occur, but the products of this reaction do not have a mechanism for recycling back to BH$_4$. 
**Figure 7 - Uncoupled eNOS due to oxidative stress.** During oxidative stress, as occurs during ischemia/reperfusion injury, BH$_4$ is converted into BH$_2$ (or XPH$_2$) and can no longer participate in stabilization of the superoxy-heme intermediate. O$_2$” disassociates from the heme and is able to modulate proteins.
Figure 8 – NADPH as a central molecule in multiple reactions. In the heart NADP$^+$ is reduced via glucose-6-phosphate dehydrogenase (G6PD); forming NADPH. NADPH is then used in the re-synthesis of the antioxidant, glutathione (GSH). In addition to its role in antioxidant production, NADPH is also the first electron donor for NOS dependent NO-synthesis. NADPH is the primary substrate for NADPH oxidase, which catalyzes the formation of superoxide ($O_2^-$). Finally, NADPH is used in the reduction of BH$_2$ back to BH$_4$ via the enzyme dihydrofolate reductase (DHFR).
Figure 9 - NADPH as a substrate in glutathione synthesis. Oxidants are scavenged via Glutathione peroxidase (GPx), which results in the production of the glutathione dimer (GSSG). The glutathione dimer is converted back to the antioxidant GSH via glutathione reductase, where NADPH is used as the reducing equivalent. The bioavailability of NADPH is dependent on the function of Glucose-6-phosphate dehydrogenase. The product 6-phosphogluco-δ-lactone proceeds into the pentose phosphate pathway.
CHAPTER 2

Ischemia/reperfusion injury results in depletion of NADPH in the whole heart as well as in the coronary endothelium, resulting in a reversible form of endothelial dysfunction.

2.1 Introduction

Previously in our laboratory it was illustrated that the loss of eNOS function can be achieved without damage to the enzyme itself, but instead could be attributed to loss of the cofactors/substrates of the enzyme [108]. More recently our lab showed that the loss of eNOS function, particularly in IR injury, could be partially explained by loss of the critical cofactor BH₄ [62]. Furthermore, in the Dumitrescu study, BH₄ repletion in the setting of the in vitro eNOS activity assay was able to restore IR damaged eNOS [62]. However, these results did not fully translate to the ex vivo isolated heart, where BH₄ repletion only partially restored eNOS dependent coronary flow [62]. The partial restitution has led to this dissertation work which is predicated on the hypothesis that NADPH, an eNOS substrate, is depleted as a result of IR injury, and that this depletion is the reason BH₄ is only able to partially restore eNOS dependent coronary flow after IR injury.

As previously stated, NADPH is a critical substrate of eNOS; as it is the first electron donor leading to the formation of the heme-superoxy intermediate [11-13]. Thus, without NADPH, eNOS cannot form NO [11]. In the endothelial cell, IR injury results in
profound oxidative stress and in this environment is it plausible that the reduced nature of
NADPH may leave it vulnerable to becoming oxidized and unavailable for NO synthesis.

In this chapter we will follow the pyridine nucleotides throughout IR injury utilizing the global ischemia and reperfusion isolated heart model (described in detail in materials/methods section). The measurements of pyridine nucleotide levels in isolated hearts after various injurious stimuli will be accomplished by high-performance liquid chromatography (Fig 10). Previous to the method developed by Klaidman et al., the ability to resolve both oxidized and reduced pyridine nucleotides on one chromatogram did not exist [109]. However, as Klaidman et al. demonstrated, by reacting potassium cyanide (KCN) with the oxidized pyridine nucleotide ring; the result is a rapid addition of cyanide at the 4 position of the nicotinamide ring (fig 11), resulting in a fluorescent product which is visible at the same emission/excitation (330/460) as the naturally fluorescing reduced counterparts [109]. Thus, any inter-conversion between reduced/oxidized or phosphorylated/non-phosphorylated pyridine nucleotides during IR injury will be easily visible within the chromatogram and offer further insights on if/how NADPH levels are fluctuating.

2.2 Materials and Methods

2.2.1 Isolated Heart Experiments

Male, Sprague-Dawley rats weighing 275-300 grams were used for these experiments. Rats were given an intraperitoneal (ip) injection of pentobarbital (50mg/kg) and hearts were excised and cannulated via the aorta and perfused in a retrograde manner
with kreb's buffer (119 mM NaCl, 17 mM Glucose, 25 mM NaHCO₃, 5.9 mM KCl, 1.2 mM MgCl₂, 2.5 mM CaCl₂, 0.5 mM NaEDTA). A small balloon connected to a pressure transducer (ADInstruments, Colorado Springs, CO), was placed in the left ventricle and measured left ventricular developed pressure (LVDP), systolic pressure (ESP), diastolic pressure (EDP), heart rate (HR) and the rate of changes of pressure over time (dP/dt). A flow probe (Transonic, Ithica, NY) measured coronary flow (CF). Isolated hearts were subjected to a multitude of injurious stimuli: no ischemia, 10, 20, and 30 minutes of ischemia as well as 30 minutes of ischemia with 10, 20, and 30 minutes of reperfusion. Hearts undergoing no injury were perfused constantly for 60 minutes, while those undergoing global ischemia or IR were first perfused for 20 minutes allowing equilibration before undergoing injury (fig 13). After the designated injury, hearts were removed from the Langendorf prep and freeze-clamped and stored in liquid nitrogen.

2.2.2 HPLC determined levels of whole-heart pyridine nucleotides in ischemia and IR injury.

For NAD(H) and NADP(H) detection via HPLC the method from Klaidman et. al. [109] was altered for frozen hearts. Hearts were homogenized with ice-cold buffer consisting of 0.2 M KCN, 0.06 M KOH and 1 mM DTPA, and centrifuged at 16,000 x g (2 times; 5 min) with chloroform. The KOH supernatant was collected and further purified using Costar Spin-X 0.45 µm pore size. Effluents from filtered homogenates were diluted 1:5 with mobile phase (0.2 mM Ammonium Acetate/4% Methanol; pH 5.8) and injected onto a TSKgel column ODS-80TM (25 cm x 4.6 m) (Supelco, St. Louis,
MO). Separation was achieved with a flow rate of 1.0 mL/min and methanol gradient (0.2% per minute for 25 minutes). Analytes were detected via fluorescence spectroscopy (wavelength ex. 330; em. 460).

2.2.3 Isolated heart experiments for endothelial levels of NADP(H)

Isolated hearts (as excised in 2.2.1) were subjected to either constant perfusion (control) or 30 minutes of IR. After 60 minutes of equilibration control hearts received a 1 second bolus of 0.25% Triton X-100 through a side-arm to the heart; permeablizing the endothelium (as previously described [108]). The effluent was collected for 3 1-minute intervals into separate eppendorf tubes. The heart was then allowed to re-equilibrate for 30 minutes before administering 100 mM histamine to determine effectiveness of endothelial permeabolization. Hearts which showed dilation with histamine infusion were omitted from the study. Prior to HPLC preparation effluent was tested with a UV/Vis spectrophotometer (Cary 50 Bio, Varian, Santa Clara, CA). Successful samples emitted strong signals at wavelengths 260 and 340 (for NAD(P)⁺ and NAD(P)H respectively), however samples which were contaminated with α/β bands of heme were omitted as extensive damage to the heart resulted in efflux of myocyte specific proteins (ie. myoglobin). For injured hearts Triton X-100 was administered in a 1 second bolus after 30 minutes of IR and the effluent was collected for 3 1-minute intervals into separate eppendorf tubes (fig 14).

Since samples contained Triton X-100, it was necessary to purge the samples of the strong detergent prior to measuring NADP(H) levels via HPLC. This was achieved by
adding in a 1:4 dilution Surfactaway (BioTech Support Group, Monmouth JCT, NJ), a concentration which removes ~94% of detergent from the sample. Samples were then concentrated using a speed Vac (Thermo Scientific, Asheville, NC) overnight. The following day samples were resuspended in 250 µL of KCN buffer (buffer contents described above), after which, Surfactaway was again added at a 1:4 dilution to ensure maximum removal of Triton X-100. Once treated for detergent removal and concentrated; samples were prepared as stated above for HPLC measurements of NADP(H).

2.2.4 Repletion of NADPH and BH₄ in the isolated heart.

Isolated hearts (as excised in 2.2.1) were subjected to 30 minutes of ischemia followed by 30 minutes of reperfusion, and either treated for 10 minutes with empty liposomes (25mg/ml), liposomal NADPH (175µM), liposomal NADPH (175µM) +BH₄ (50µM) or liposomal NADPH (175µM) + L-NAME (1mM) at 1/100th coronary flow rate for a final concentration of 17.5µM for NADPH and 5µM for BH₄. After treatment the hearts were allowed to re-equilibrate for 10 minutes before ending experiment (fig 15).

Phosphatidylcholine (PC) from soy bean was used for liposomal preparation. For reasons which remain unexplained, when liposomal preparations used PC derived from egg yolk the liposomes behaved very differently and exhibited a propensity to coagulate; limiting their efficacy in delivering contents. Liposomes for repletion studies were prepared by first solubilizing PC (from soy bean) in chloroform for a final concentration of 25 mg/mL (it is also possible to use another organic compound such as ethanol or
DMSO for solubilization, however for consistency chloroform was always used). The solution was subsequently dehydrated under argon gas; forming a thin layer of PC on the wall of the glass jar. Krebs buffer (as described in 2.2.1) containing 175 µM NADPH, 50 µM BH$_4$ or a combination of both was then used to rehydrate the PC. The glass jar was then placed on a long roll jar mill (U.S. Stoneware, East Palestine, OH) for 1 hour, which allowed for micelle formulation of PC around NADPH/BH$_4$, forming a milky white solution. Liposomal preparation was passed through a membrane extruder ensuring the size of liposomes was less than 200 nanometers, after which, the liposomal formulation was ready for administration to isolated heart.

2.2.5 Statistics

Results were expressed as means ± SE. Statistical significance (P < 0.05) was determined by ANOVA (followed by Newman-Keuls test) for multiple groups. Paired or unpaired t-tests were used for comparison between two groups.

2.3 Results

2.3.1 Effects of ischemia and reperfusion on whole-heart levels of NADP(H)

NADPH levels trend upwards slightly in early ischemia (59.91 nmol/gram tissue ± 3.39); however by mid to late ischemia these levels return to pre-ischemic values. During the same time period NADP$^+$ levels declined briefly (13.64 nmol/gram tissue ± 1.90) but then return to near pre-ischemic levels (18.05 nmole/gram tissue ± 1.34). This tight correlation between NADPH and NADP$^+$ suggests inter-conversion between the two
molecules, which can occur upon activation of endogenous intracellular antioxidant systems (see 1.6.1). During late reperfusion this tight regulation is lost and NADPH levels decline ~50% (29.87 nmol/gram tissue ± 3.90 P<0.05, when compared to pre-ischemic levels), while the oxidized form, NADP⁺, drops by approximately 70% (7.60 ± 1.55 P<0.05, when compared to pre-ischemic levels) (fig 16 left).

2.3.2 Ratio of NADP(H) during ischemia/reperfusion injury

Figure 16 (right) depicts a very distinct intracellular shift in the phosphorylated pyridine nucleotide pool from oxidized to reduced in both the early periods of ischemia and reperfusion. Both of these shifts correlate with reported free radical generation in ischemia [88] and the more pronounced free radical burst in reperfusion [61, 88, 110]. Interestingly, these shifts are transient, suggesting they may be unsustainable and could prove limiting to enzymes using NADPH as an electron donor.

2.3.3 Effects of ischemia/reperfusion on whole-heart levels of NAD(H)

Our results show that in the normal heart tissue, intracellular levels of NAD(H) are consistently 10x the levels of the phosphorylated pyridine nucleotides. The unphosphorylated pyridine nucleotides (NAD⁺/NADH) response to ischemia/reperfusion is much different than the phosphorylated pool. At the onset of ischemia NADH rises dramatically (10 min ischemia: 334.4 nmol/gram tissue ± 60.2 P<0.05 when compared to pre-ischemic levels) and is sustained throughout the ischemic period. Conversely NAD⁺ during the same time period (10 min ischemia: 308.5 nmol/gram tissue ± 59.1 P<0.05
when compared to pre-ischemic levels) falls at a proportional rate. As flow is restored to the heart the unphosphorylated pyridine nucleotides return to, but never approach preischemic levels (30 min reperfusion: NAD$^+$ 298.1 nmol/gram tissue ± 20.2; NADH 125.0 nmol/gram tissue ± 10.1) (fig 17 left).

2.3.4 Ratio of NAD(H) during ischemia/reperfusion injury

The large fluctuations in the unphosphorylated pool are distinct from the non-phosphorylated pool, which only displays minor shifts between reduced and oxidized forms during ischemia. At the onset of ischemia the NAD$^+$/NADH pool shifts towards NADH, and this becomes even more pronounced as ischemia progresses. This is likely due to the inability of the electron transport chain to effectively use NADH during mitochondrial respiration. Upon reperfusion the pool shifts back towards NAD$^+$ but never to pre-ischemic values (fig 17 right).

2.3.5 Effects of ischemia/reperfusion injury on endothelial levels of NADP(H)

To measure IR effects on pyridine nucleotides within endothelium a modified version of the method developed by Girladez et al. was used [108]. In the first three minutes after Triton X-100 treatment NADP(H) efflux levels were high (0.456 ± 0.17 nmoles NADP(H)/min). The same Triton X-100 treatment was performed on isolated hearts undergoing 30 minutes of IR injury and during the same time period only nominal levels of NADP(H) were detected in the effluent (0.069 ± 0.05 nmoles NADP(H)/min).
The unphosphorylated pool, NAD(H), undergoes a similar but less drastic decline during IR. Furthermore this small decline was not shown to be significant (P=0.09) (fig 22).

2.3.6 Repletion of eNOS cofactors/substrates

The physiological significance of lost NADPH was demonstrated by repletion of the eNOS substrate 30 minutes post-reperfusion, when levels where shown to be depleted. Liposomal-NADPH delivered at 30 minutes post-reperfusion yields a 30.87 ± 3.23% rise when compared to baseline coronary flow. When combined with BH₄ coronary flow increases to 38.97 ± 5.77%, or almost 4 times the amount when BH₄ alone is repleted (9.19 ± 2.01%). Co-administration of NADPH and L-NAME, an eNOS inhibitor, shows detrimental effects to coronary flow.

2.4 Discussion

The data derived from this study answers the questions left from the work presented by Dumitrescu et al. Namely, why BH₄ repletion does not fully restore coronary flow after IR injury in the ex vivo isolated heart. These results show that IR injury causes a two-fold hit on eNOS enzymatic function, first during ischemia BH₄ levels drop dramatically as exhibited by Dumitrescu et al. [62]. This work for the first time demonstrates a second hit which occurs during reperfusion, where depletion of NADPH levels, mainly within the endothelium results in eNOS dysfunction, which would persist even with BH₄ repletion.
2.4.1 The fluctuating levels of NADP(H) in response to ischemia/reperfusion injury.

The whole heart levels of NADP(H) offer a very insightful view of how the molecule is being utilized during IR injury. In early ischemia (10 min) there is a slight increase of NADPH met with an equal decline in NADP⁺, suggesting direct conversion of NADP⁺ to NADPH. However, of greater question is why these levels are not continually elevated during IR injury (explored further in chapter 3).

During ischemia the levels of NADPH and NADP⁺ are very tightly bound to one another, however during reperfusion this relationship is lost. Within the first 10 minutes of reperfusion there is a drop in NADP⁺ but not in NADPH. A decline of NADP⁺ would not allow for rapid recovery of NADPH via Glucose-6-Phosphate dehydrogenase (G6PD). G6PD is the primary synthesizer of NADPH from NADP⁺ and participates in the pentose phosphate pathway (the contribution of this enzyme to NADPH levels if further explored in chapter 3). The meticulous tracking of NADPH via HPLC throughout IR injury reveals a dramatic decline of NADPH during reperfusion (fig 16). Whether this decline is sufficient to cause endothelial dysfunction lead to repletion studies where it was determined if depleted NADPH was limiting in eNOS dependent NO production.

2.4.2 A stark contrast in the behavior between phosphorylated and non-phosphorylated pyridine nucleotides in response to ischemia/reperfusion injury.

The unphosphorylated pyridine nucleotide pool, NAD(H), maintains a much different response to IR than the phosphorylated pool. NADP(H) levels are predominately linked to redox homeostasis and pentose phosphate synthesis, where
NAD(H) levels are linked predominately to oxidative phosphorylation and the synthesis of ATP. During ischemia the levels of oxygen plummet and the cell can no longer undergo respiration; resulting in the build-up of NADH molecules, as demonstrated in figure 17 (left). However, during reperfusion the reintroduction of oxygen allows for respiration to continue, however as demonstrated by Quarrie et al. proton leakage from complex 1 results in limited function of the electron transport chain, and NADH is not efficiently utilized [83]. This is demonstrated in my work as elevated NADH levels persist even though oxygen is restored during reperfusion.

Interestingly, there is a notable decline in NAD\(^+\) levels in ischemia, but this becomes even more pronounced in late-stage ischemia (fig 17; left). This may partially be explained by cessation of respiration, but also by poly-ADP polymerase 1 activation (PARP-1) [111]. The results of this study suggest that the modulation induced by IR injury on NAD(H) and NADP(H) are of distinct mechanisms and do not directly influence one another (i.e. inter-conversion between NAD(H) and NADP(H)).

### 2.4.3 Oxidative stress and ischemia/reperfusion injury

The susceptibility of BH\(_4\) to oxidation has been elegantly described in literature; in the oxidative stressed environment of IR injury BH\(_4\) is modified. Since NADPH is a reduced molecule, it then lends to the possibility that IR is acting in the same way and potentially oxidizing the molecule making its bioavailability limiting in eNOS-dependent NO formation.
Indeed, when looking at the ratio of NADP$^+$/NADPH the modulation in levels coincide with the modest release of free radicals during ischemia [88] and the free radical burst of reperfusion [61, 88, 110]. However, at the time points of these early free radical bursts (i.e. 10 min IR) NADPH levels are not declining. Only after 20 minutes of IR injury do the levels of NADPH drop significantly (29.38 nmol/gram tissue ± 3.71 P<0.05 when compared to pre-ischemic levels), well after the free radical burst of reperfusion which occurs within the first few minutes of reperfusion [61, 88, 110].

It is possible that reperfusion results in a general washout of molecules from the intracellular stores, resulting in the lowered levels of NADPH. While it is possible that the expansive disruption of IR may result in NADP(H) efflux, the results of the this work would suggests that this would only account for small fraction of the lost NADP(H). General washout of pyridine nucleotides would result in equal depletion of all pyridine nucleotides, however we see that this is not the case when comparing NAD(H) to NADP(H). Even within the phosphorylated pool, the decline of NADPH (~50%) is significantly less than NADP$^+$ (~70%). Furthermore within the endothelium there is a nominal decline in NAD(H) levels, but a large decline in NADP(H) levels, the lack of uniformity in the decline of the endothelial molecules is not suggestive of washout. The targeting of one pyridine nucleotide over another would suggest the mechanism for the depletion of the eNOS substrate, NADPH, during reperfusion is more complex than simply oxidation to NADP$^+$ or washout and is the basis for the work explored in chapter 3.
2.4.4 Repletion of eNOS cofactors and substrates as a therapy for endothelial dysfunction.

As discussed in section 1.3.1 eNOS functions in the coronary arteries via NO which precipitates vasodilation and increased vascular flow. The isolated heart allows for measurements of not only cardiac function, but also coronary flow. Thus significance of NADPH depletion can be measured by its influence on coronary flow and cardiac function.

The most clinically significant outcome from these studies was the effect NADPH had on coronary flow after 30 minutes of ischemia and 30 minutes of reperfusion; where infusion of NADPH (17.5µM) resulted in a large and sustained increase in coronary flow (fig 18). This increase was determined to be eNOS specific through the co-infusion of L-NAME (an eNOS inhibitor), which resulted in loss of the vasodilatory effects promoted by NADPH. When infusing both NADPH and BH₄ (17.5µM and 5µM respectively) there was an additive effect to the recovery of coronary flow, although not significantly different from NADPH infusion alone. NADPH proved to be more efficient at restoring coronary flow than BH₄, which was infused at the concentration used by Dumitrescu et al. (fig 18). Surprisingly, the improvement in coronary flow did not result in a concurrent improvement of cardiac function fig (21).

This dichotomy in recovery may be explained through examination of the experimental design. The goal of this experiment was to determine if NADPH was potentially limiting in eNOS dependent NO production. In section 2.3.1 this was determined to be during reperfusion at a time point in excess of 20 minutes. Figure 18
shows recovery of coronary flow at 30 minutes of reperfusion, but earlier time points where not measured. It may be that at 30 minutes post-reperfusion the damage to myocardium is set and subsequent improvement of coronary flow may not matter. The lack of early time points is a limitation to this study, which leaves the question whether earlier infusion of NADPH could result in improved cardiac function. However, figure 16 clearly showed NADPH levels to remain near pre-ischemic values during early ischemia; therefore it may prove that NADPH infusion during this time-point may not improve endothelial function.

Finally, the difference in recovery may also be explained though “stunning”, which is a phenomenon where myocardial recovery after a coronary occlusion can at first be abnormal but over time can recover and display normal contractile behavior. The stunning phenomenon can last up to two weeks, which again highlights the limitation of the isolated heart which is focused more on the acute setting of reperfusion injury. However improvement of coronary flow may help recover stunned myocardium, leading to better contractile function. The effect of stunned myocardium would be better measured in an in vivo ligation model where time points of reperfusion could be measured out to 24 and 48 hours (this is further explored in chapter 5).

2.4.5 The whole heart vs. endothelial levels of NADP(H)

Again, while NADPH infusion mitigated endothelial dysfunction, this did not result in improvement of cardiac function (fig 21). The specificity in the effect on coronary flow but not on cardiac function led us to explore the endothelial dependent
levels of NADP(H) as a result of IR injury. Indeed, the levels of endothelial NADP(H) appeared to be much more influenced by IR injury, where levels were found to be ~75% depleted (fig 20). Thus it appears that IR injury results in an endothelial targeted depletion of NADP(H), resulting in reversible endothelial, but not cardiac, dysfunction.

2.4.6 Conclusion.

NADPH is a substrate for many enzymes; most notably NADPH oxidase, which has been shown to be the primary source of superoxide in endothelial cells [112, 113]. Given this; it becomes less clear as to why exogenous NADPH would lead to alleviation of endothelial dysfunction, and not increase its detriment. However, in previous studies when NADPH or NADH was added in amounts comparable to the present study, 0.1mM, it led to only a modest increase of superoxide production [112]. Furthermore it was demonstrated elsewhere [113] that levels of NADPH infused in our setup even with superoxide dismutase (SOD) inhibitors would not lead to an abundance of NADPH oxidase derived superoxide. For superoxide levels to become overwhelming the infusion of NADPH would have to be 1mM, or almost 100 times the levels used in this study [113]. Thus it is not surprising that the infused levels of NADPH for this work did not further exacerbate eNOS dysfunction.

The conclusion of these studies illustrate that NADP(H) is depleted during IR injury, and that this depletion while notable at the whole heart level, becomes pathological within the endothelium of the coronary arteries. eNOS dysfunction perpetuated by IR injury is alleviated by repletion of lost NADPH. In the following
chapter we will explore the mechanism for NADPH depletion during IR and determine whether inactivation of this mechanism can salvage NADPH and eliminate eNOS dysfunction.
Figure 10 - Chromatograms of pyridine nucleotides throughout ischemia/reperfusion. Homogenates from isolated hearts were prepared for HPLC by the method outlined in section 2.2.2. Via reaction with KCN, all pyridine nucleotides fluoresce at the wavelength ex. 330; em. 460 and are visible on one chromatogram.
Figure 11 - Oxidized pyridine nucleotide reaction with KCN. Here NAD$^+$ and NADP$^+$ are shown in the reaction with KCN. The reaction yields a rapid addition of cyanide at the 4 position of the nicotinamide ring. NAD$^+$ (bottom) has same structure as NADP$^+$, but without the phosphate group on the 2 position of the ribose ring.
Figure 12 - Linear regression of pyridine nucleotide peaks. Peaks were quantified by using known quantities of standards and plotting the unknown peaks during ischemia and reperfusion on a linear regression line.
Figure 13 - Protocol for isolated heart studies aimed at determining levels of pyridine nucleotides during ischemia/reperfusion injury. Hearts were divided up into one of three groups: Control (no injury), Ischemia and Ischemia/Reperfusion. Hearts in Control group received no injury and were perfused for a constant 60 minutes. Hearts in Ischemia group were allowed to equilibrate for 20 minutes before being subjected to 10, 20 or 30 minutes of ischemic injury. Finally, hearts in Ischemia/Reperfusion group underwent 20 minutes of equilibration and 30 minutes of ischemia, followed by either 10, 20 or 30 minutes of reperfusion.
Figure 14 - Protocol for measuring endothelial levels of NADP(H). The method of Giraldez et al. was modified for these studies. Isolated hearts undergoing 60 minutes of constant perfusion or 30 minutes of ischemia/reperfusion injury were infused with a bolus of 0.1% Triton X-100 and the effluent collected. The effluent was then processed for HPLC where levels of NADP(H) were measured.
Figure 15 - Protocol for repletion of NADPH/BH$_4$. Isolated hearts received 30 minutes of IR injury. 30 minutes post reperfusion 1 of 5 treatments was given for 10 minutes: empty liposomes (25mg/ml), liposomal NADPH (175µM), liposomal NADPH (175µM) + BH$_4$ (50µM) or liposomal NADPH (175µM) + L-NAME (1mM) at 1/100$^{th}$ coronary flow rate.
Figure 16 - Levels of NADP(H) during IR injury. Isolated hearts were subjected to global ischemia for either 10, 20 or 30 minutes, or 30 minutes of global ischemia followed by 10, 20 or 30 minutes of reperfusion. During each time point levels of the pyridine nucleotides (NAD^+, NADH, NADP^+ and NADPH) were measured via HPLC. (Right) During early ischemia NADPH increases slightly; however by late ischemia the levels of NADPH fall back to pre-ischemic levels. Upon reperfusion, levels do not change until 20 minutes of ischemia, when levels fall a dramatic ~50% of pre-ischemic levels. The oxidized form (NADP^+) behaves differently as a result of ischemic/reperfusion injury, in that levels drop immediately upon reperfusion. (Right) Interestingly when examining the entire pool the ratio of oxidized to reduced NADP(H) shifts in early ischemia and again in early reperfusion. *P<0.05 when compared to pre-ischemic values.
Figure 17 - Levels of NAD(H) during IR injury. (Left) In normal tissue, the unphosphorylated pool is kept in the oxidized form, as levels of NADH are nearly undetectable. During ischemia levels of NADH immediately rise as the electron transport chain ceases due to lack of oxygen. During reperfusion levels of NAD$^+$ and NADH approach, but never reach pre-ischemic values. (Right) NADH dominates during early ischemia, but the most dramatic shift in levels occurs during late reperfusion.
Figure 18 - Repletion of NADPH post-ischemia – Isolated hearts were subjected to 30 minutes of global ischemia followed by 30 minutes of reperfusion; at which point they were administered one of four treatments; NADPH (176µM) with BH4 (50µM), NADPH (176µM) only, BH4 (50 µM) only and NADPH (176 µM) with L-NAME (1mM). All treatments were administered using liposomal formulation (25mg/ml) which ensured that NADPH and BH4 crossed the lipid bilayer as well as prevented oxidization of these molecules. (Top) Raw coronary flow tracings at 30 minutes post-reperfusion demonstrated a large and sustained increase in flow when infusing NADPH with and without BH4. NADPH repletion with L-Name shows a negative effect on coronary flow. (Bottom) Quantified data shows that treatment of IR hearts with NADPH versus BH4 alone resulted in coronary flow increase which was more than 3.5 times greater than when using levels of BH4 repletion previously reported. When administering NADPH along with L-NAME, an eNOS inhibitor, the recovery in coronary flow is lost, suggesting the benefits of NADPH are largely a result of endothelial recovery.*P<0.05 when compared to BH4. P=0.2 when comparing NADPH and NADPH+BH4 infusion.
Figure 19 – Representative chromatograms for endothelial NADP(H). Chromatograms were baseline corrected to reduce background noise using MATLAB software (Math Works, Natick, MA). Peaks eluted at similar time points to whole-heart homogenates, and displayed a noticeably decreased NADP(H) peak size when comparing control and IR chromatograms. This decline became less noticeable when comparing NAD(H) peaks.
Figure 20 – Effects of IR Injury on Endothelial NADP(H) – While NADP(H) is depleted in the whole heart, this depletion is more dramatic in the endothelium of the isolated heart. NADP(H) collected from the effluent of Triton X-100 treated control hearts (0.41 nmoles NADP(H)/min ± 0.16) yielded nearly 3 times the amount detected from the effluent of IR hearts receiving the same Triton X-100 treatment (0.07 nmoles NADP(H)/min ± 0.05). (When comparing values of Control to IR *P<0.05)
Figure 21 – Cardiac dysfunction persists with NADPH infusion. Data is presented as percent of pre-ischemic values (PI). While benefits to coronary flow were immediate; NADPH repletion did not illustrate any benefit as measureable by cardiac parameters.
Figure 22 – Endothelial levels of NAD(H). Levels of endothelial NAD(H) are slightly depressed after IR injury. However this decline of NAD(H) was not shown to be significant. (P=0.09).
CHAPTER 3

Influence of CD38, as an ADP-ribosyl cyclase, on eNOS function during Ischemia/Reperfusion injury

3.1 Introduction

The focus of this chapter will concentrate on the mechanism(s) for NADP(H) depletion, which was demonstrated in the previous chapter to be critical in eNOS function post-IR injury. The mechanism as discussed in chapter 2, is likely more complex than simple oxidation or washout, and possibly consists of a problem involving underproduction, overconsumption or a combination of both.

To explore the possibility that NADP(H) depletion is the result of underproduction we examine the enzymatic function of Glucose-6-Phosphate dehydrogenase (G6PD), which regulates the intracellular levels of phosphorylated pyridine nucleotides by catalyzing the reduction of NADP+ to NADPH. While other enzymes can catalyze this reaction; Jain et al. demonstrated that G6PD was the most important during IR in the myocardium [114].

While regulation of production is limited to G6PD in the isolated heart, our attempts to understand which enzymes could catalyze reactions that would shunt NADP+ into products which cannot be reduced to NADPH was much more complex and yielded three different enzymes of interest. The first, NAD kinase (NADK), has been described in a number of mammalian tissues [115-117], and catalyzes the following reaction:
ATP + NAD$^+$ $\rightarrow$ NADP$^+$ + ADP

In the setting of IR the heart is starved for ATP (see section 1.5.1.1), therefore we hypothesized that NAD kinase might also catalyze the reverse reaction in an effort to exhaust all intracellular stores of ATP. This reaction would explain the depletion of NADP$^+$ seen within the whole-heart as described in the following reaction:

ADP + NADP$^+$ $\rightarrow$ ATP + NAD$^+$

However, Apps and Narin demonstrated in NADK purified from pigeon liver the reversibility of the reaction, and reported an extremely low value for $K_{eq}$ (30x10$^{-7}$) [118]. Therefore, the likelihood that the enzyme could catalyze the backwards reaction would be nearly non-existent in our model; leaving this a nonviable path for exploration.

Moving on from NAD kinase we turned our attention to an emerging field involving the enzyme NADP$^+$ phosphatase (NADPase). The recent discovery of an archaeon (*Methanococcus jannaschii*) NADPase [119] has led to the postulation that within other organisms this enzyme may exist [117]. Indeed NADPase activity has been described within the mitochondria of rat liver [120], however the enzyme itself has yet to be isolated. Regardless, the location of the enzyme offers a possible unique mechanism where release of the enzyme from the mitochondria could follow lysis, as is often found as a result of IR injury [76, 79]. In this chapter we will explore the possibility of NADP$^+$
phosphatase existence within the isolated rat heart, and determine whether activity of this enzyme leads to depletion of NADP(H).

The final and equally novel mechanism involves CD38 and its possible influence on eNOS function. In addition to superoxide production and endothelial dysfunction, IR injury also brings about Ca\textsuperscript{2+} overload, which has been attributed to, in part by activation of CD38 [121, 122]. CD38 as an ADP-ribosyl cyclase is central to this thesis work; however CD38 posses other functions which make it a multi-dimensional and complex enzyme. Therefore in the interest of comprehension and completeness we will first address these functions so that results from this work are better appreciated.

### 3.1.1 Structure and function of the ADP-ribosyl cyclase family

Originally identified as an antigen marker on B-Cells [123], CD38 was later found to contain sequence homology with ADP-ribosyl cyclase (ARC) of *aplysia californica* [124]. Since this discovery, ADP-ribosyl cyclase has been found in a number of tissues across kingdoms and its activity has been linked to a number of cellular responses from stress response in plants to cell proliferation, chemotaxis, synaptic depression and cell proliferation in animal cells [125-129]. CD38, CD157 and ARC from *aplysia californica* comprise the family of ADP-ribosyl cyclases, in which all maintain the same active site of TLEDTLGY, and all catalyze the formation of cADPR from NAD\textsuperscript{+} [130]. CD38 and CD157 are differentiated in their ability to hydrolyze cADPR and catalyze the base exchange reaction with nicotinic acid to form nicotinic acid adenine
dinucleotide phosphate (NAADP) (fig 23). The multifunctional characteristics of CD38 were realized when the crystal structure of ARC was attained.

The crystal structure of ADP-ribosyl cyclase shows an active site which sits in a central pocket of the overall structure (fig 24). Interestingly, there exists 12 cysteine residues which are paired; of these residues, 10 are conserved across the ARC family [130]. The pair which is not conserved is found in both CD38 and CD157; the additional cysteines are significant as they are involved in hydrolase function (explored later) [130].

Critical residues of ARC have been defined through mutagenesis; Glu 179 is likely the catalytic residue, as changing it to aspartate eliminates all enzymatic activities [131]. Within the active pocket there exits two tryptophan residues (Trp 77 and Trp 140) which are responsible for molding the substrate in the active pocket [131]. The homologous catalytic residue in CD38 is Glu 226 [132]. CD38 also maintains tryptophan residues within its active site (Trp 125 and Trp 189) where they act as Trp 77/140 of ARC and mold the substrate within the active pocket [132].

As stated previously, CD38 also catalyzes the production of NAADP, which has been shown to occur under acidic conditions [133]. The crystal structure reveals an abundance of acidic residues at the active site pocket (fig 25), which suggest the possibility that they exert electrostatic repulsion to the nicotinic acid group of NAADP [134]. In an acidic environment, both the nicotinic acid group and the active site pocket would be protonated [135]. Figure 25 illustrates the NAD analogue, NMN, bound to the active site, where the molecule binds very closely to two acidic residues, Glu 146 and Asp 155. Mutagenesis of Glu 146 (to Gly or Ala) or Asp 155 (to Gln or Asn), eliminates
the electrostatic repulsion and allows for the base exchange reaction of NAADP at an alkaline pH, thereby confirming the pH regulation of NAADP synthesis.

Finally, the enzymatic functions of ARC can be modified by the cysteine residues. Site mutagenesis of Cys 119 and/or Cys 201 results in loss of hydrolase activity in CD38/157. Furthermore, when mutating Lys or Glu of ARC to mirror CD38/157, there is a gain of function, allowing for ARC to hydrolyze cADPR. Additionally, it has been demonstrated that DTT incubation (1-3mM) [136] with CD38 resulted in decreased function; further highlighting its redox sensitivity as mentioned above.

3.1.2 CD38 as an ARC and its role in IR

The primary product of ARC/CD38 is cADPR, which has been demonstrated repeatedly as a potent signaler for Ca\(^{2+}\) release via intracellular stores through activation of the ryanodine receptor (RyR)[135, 137, 138]; as stated previously this mechanism has been shown to be activated during IR [121, 122]. While activity of CD38 consistently is measured by production of cADPR, the enzyme also has the ability to utilize NADP\(^+\) and form both 2\(`\)-P-cADPR [127, 139] and NAADP [140]. Both 2\(`\)-P-cADPR and NAADP have been shown to release Ca\(^{2+}\) via intracellular stores, where 2\(`\)-P-cADPR acts in a manner which mirrors cADPR [127, 139]; NAADP behaves differently and acts on lysosomal stores of Ca\(^{2+}\) [141, 142]. Thus, in the IR setting, it is possible that CD38 is utilizing NADP\(^+\) and converting it into a molecule which cannot be recycled back into NADPH, thus limiting its bioavailability for eNOS; resulting in endothelial dysfunction.
3.2 Materials and Methods

3.2.1 Measurement of Glucose-6-Phosphate Dehydrogenase (G6PD) activity in ischemia/reperfusion injury.

Isolated hearts (as excised in 2.2.1) subjected to either constant perfusion (control), ischemia (30/60min) or ischemia/reperfusion (30/60min); were homogenized in ice cold 50 mM phosphate buffer (pH 7.0). The samples were then centrifuged for 10 minutes at 14,000 x g. The supernatant was collected and the pellet discarded. Half of the supernatant was stored and the other half was run through a HiTrap™ desalting column (GE Healthcare, Piscataway, NJ), removing all small molecules involved in G6PD activity. In separate reactions protein from unfiltered homogenate as well as homogenate filtered through desalting columns (100 μg) were added to the reaction mixture (55 mM Tris-HCl; pH 8.0, 3.3 mM MgCl₂, 3.5 mM glucose-6-phosphate, and 0.2 mM NADP) for 5 minutes. Activity was measured as formation of NADPH and quantified using UV/VIS (Cary 50Bio, Varian) at 340 nM in a temperature controlled cuvet (30 °C).

3.2.2 NADP⁺ phosphatase activity in isolated heart

Isolated hearts were subjected to either constant perfusion (control) or 30 min of IR. After injurious stimuli, hearts were taken down from Langendorf prep and minced in ice-cold HEPES buffer (3mM HEPES, 70 mM sucrose and 230 mM mannitol). Minced hearts were then homogenized using a Polytron tissue grinder (Brinkmann Instruments, Westbury, NY) (5x; 3sec) in ice-cold modified HEPES buffer (3mM HEPES, 70 mM sucrose, 230 mM mannitol, 0.2 mM EGTA and 4 mg/mL BSA). To ensure proper
homogenization, samples were homogenized once again using dounce homogenizers. Once completed, homogenates were centrifuged at 500 x g for 10 minutes, after which the supernatant was collected and the pellet discarded. The supernatants were passed through a Hi-Trap™ desalting column (GE Healthcare, Piscataway, NJ), to ensure removal of all endogenous pyridine nucleotides. 100 µg of protein from homogenate were added to 20 mM Tris buffer. The reaction was started by adding 20 µM NADP⁺ and placed in 37°C water bath for 30 min. After 30 minutes 50 µL of the reaction mixture was added to equal parts of KCN buffer (described previously) and allowed to react for 5 min, then injected onto the HPLC column as described in 2.2.2.

3.2.3 Western Blot of CD38

Isolated hearts (as excised in 2.2.1) were subjected to either no injury (control), 30 minutes of ischemia or 30 minutes of ischemia plus 30 minutes reperfusion. Isolated hearts were subjected to either no injury (control), 30 minutes of ischemia or 30 minutes of ischemia plus 30 minutes reperfusion. Hearts where homogenized in ice-cold Tris buffer, pH 7.5 (Tris 50 mM, NaCl 150 mM, 0.5% NP-40, Na₄P₂O₇ 1 mM, NaVO₃ 5 mM, Benzamide 1 mM, NaF 1 mM). Samples were homogenized using a Polytron tissue grinder (Brinkmann Instruments, Westbury, NY) (5x; 10 sec), after which samples were kept on ice for 30 min for protein solubilization. Samples where then spun for 10 min (4°C; 13,000 x g) and the supernatants collected. Two additional high speed centrifugations were performed (100,000 x g; 1 hr) where the pellet was collected and reconstituted in the first spin and the supernatant was collected in the final spin. A
Bradford assay was used to determine protein concentration of samples. 60 µg of protein was loaded onto a 4-20% Tris-Glycine gel (Invitrogen, Grand Island, NY) under reducing conditions (100mM DTT) and subsequently transferred at room temperature for 3 hours. Blots were washed 3x with TBST (0.05% Tween) for 15 minutes, then blocked overnight in 5% non-fat blotting grade dried milk (BioRad, Hercules, Ca) in TBST. The following day anti-CD38 primary (Santa Cruz Biotechnology, Santa Cruz, Ca) was added at 1:300 concentration for 2 hours at room temperature. The blots were again washed (6x; 10 min) at room temperature in TBST. Secondary antibody (donkey-anti goat; Santa Cruz Biotechnology, Santa Cruz, Ca) was added at concentration of 1:5000 in 5% non-fat blotting grade dried milk in TBST to blots. Blots again washed (6x; 10 min) with TBST and subsequently developed using the Amersham ECL Western Blotting Detection Reagent (GE Healthcare, Piscataway, NJ).

3.2.4 Immunohistochemistry of CD38, vWF and eNOS

Isolated hearts (as excised in 2.2.1) were subjected to either continuous perfusion (control), 30 minutes of ischemia or 30 minutes of ischemia plus 30 minutes reperfusion. After the designated injury, hearts were removed from the Langendorf prep and embedded in optimal cutting temperature compound (OCT) and stored at -80ºC. OCT embedded hearts were serially sectioned using a cryostat (Leica, Buffalo Grove, IL) at -20ºC. The sections (5 µm) were attached to cover slips and fixed with 3.7% paraformaldehyde for 10 min, permeabilized with 0.25% Triton X-100 in TBST containing 0.01% Tween-20 for 5 min, and blocked for 30 min with 1% BSA in 0.01% TBST and
incubated with anti-CD38 (Santa Cruz Biotech, Santa Cruz, CA), anti-eNOS (BD Biosciences, San Jose, CA) and anti-vWF primary antibodies at a dilution of 1:300; 1:500; 1:200 respectively in 0.01% TBST containing 1% BSA for 1 hour at room temperature. Following treatment of sections with the chosen primary antibodies, they were incubated with secondary antibodies: Alexa Fluor 488 (anti-goat), Alexa Fluor 568 (anti-mouse) and Alexa Fluor 647 (anti-rabbit) (Life Technologies, Grand Valley, NY) at 1:1000 dilution, for 1 hour at room temperature. After washing with TBS-T the sections were then mounted with the mounting medium, Fluoromount-G (Southern Biotech, Birmingham, AL). Slides were then visualized using a scanning microscope Olympus FV1000 filter/spectral imaging system (Olympus, Center Valley, PA) at magnification 60x. The digital images were quantitatively analyzed for fluorescence intensities using the Olympus OIB software (Olympus, Center Valley, PA).

3.2.5 Measurement of CD38 activity from whole heart homogenates

Isolated hearts (as excised in 2.1.1) were subjected to either constant perfusion (control), 30 minutes of ischemia or 30 minutes of ischemia/reperfusion. After Langendorf prep hearts were homogenized using a Polytron tissue grinder (Brinkmann Instruments, Westbury, NY) (5x; 10sec) in ice-cold buffer (HEPES 20 mM; pH 7.3, Sucrose 250 mM, EDTA 0.1 mM, β-2 Mercaptoethanol (β2ME) 2.5 mM), after which, samples were kept on ice for 30 min for protein solubilization. Homogenates of injured and non-injured isolated hearts were first run through a HiTrap™ desalting column removing all substrates of the enzyme, then 25 μL of the homogenate was added to the
reaction mixture (Tris buffer 50 mM; pH 7.4 with 100 µM NADP^+), and allowed to run for 30 minutes in 37˚C water bath (Fisher Scientific, Pittsburgh, PA). Upon reaction completion samples were quickly removed and centrifuged for 3 minutes using Costar Spin-X 0.45 µm pore size, thereby separating enzyme from reaction substrates/products which pass through membrane. The effluent from Costar Spin-X tubes were diluted 1:3 with mobile phase (0.04 M Sodium Phosphate/1% methanol; pH 7.0) and injected onto a TSKgel column ODS-80TM (25 cm x 4.6 m) (Supelco, St. Louis, MO). Separation was achieved with a flow rate of 1.0 mL/min with buffer A (0.04M Sodium Phosphate) and buffer B (0.04M Sodium Phosphate/Methanol 1:1) gradient as follows: 1 min, 0%; 2.5 min, 0.5%; 5min, 3%; 7min, 5%; 8min, 12%; 10 min, 20%; 12 min, 30%; 20 min, 50%. (fig 30). 2’-P-cADPR and other analytes of interest were detected via UV/Vis detector (ESA model 520, Chelmsford, MA) at wavelength 254.

3.2.6 Measurement of 2’-P-cADPR during ischemia/reperfusion injury using HPLC

Isolated hearts were prepared as in section 3.2.5, but once homogenized samples differed as follows: homogenates where centrifuged at 16,000 x g (2 times; 5 min) with chloroform. The supernatant was collected and further purified using Costar Spin-X 0.45 µm pore size. The effluent from Costar Spin-X tubes were diluted 1:3 with mobile phase (0.04 M Sodium Phosphate/1% methanol; pH 7.0) and injected onto a TSKgel column ODS-80TM (25 cm x 4.6 m) (Supelco, St. Louis, MO). Separation was achieved as in section 3.2.5. 2’-P-cADPR and other analytes of interest were detected via UV/Vis detector (ESA model 520, Chelmsford, MA) at wavelength 254.
3.2.7 *Inhibition of CD38 using α-NAD in the isolated heart*

Isolated hearts were subjected to either constant perfusion (control) or 30 minutes IR. Hearts which received 30 minutes IR received either 5mM α-NAD treatment or sham treatment (Krebs buffer). 3 minutes prior to ischemia 5mM α-NAD, dissolved in krebs buffer (contents described in 2.2.1), and was administered at a rate of 10 mL/min (30 mL total) via sidearm. All parameters previously measured (2.2.1) were recorded for α-NAD effects on heart function.

3.2.8 *Measurement of endothelial NADP(H) from α-NAD treated isolated hearts*

Hearts were prepared as in 3.2.6 and endothelial levels of NADP(H) were determined via HPLC as described in 2.2.3.

3.2.9 *Statistics*

Results were expressed as means ± SE. Statistical significance (P < 0.05) was determined by ANOVA (followed by Newman-Keuls test) for multiple groups. Paired or unpaired t-tests were used for comparison between two groups.

3.3 *Results*

3.3.1 *Glucose-6-phosphate dehydrogenase activity in ischemia/reperfusion injury*

G6PD activity is unchanged across all groups when supplementing the assay with exogenous levels of glucose-6-phosphate (G6P) (fig 26 Left). However when limiting the
assay to endogenous levels of G6P, the enzyme substrate becomes limiting during 30
(2.15 µmoles NADPH/min ± 0.22; P<0.05 when compared to PI) and 60 (1.71
NADPH/min ± 0.30; P<0.05 when compared to PI) minutes of ischemia (fig 26 right).
During reperfusion the limitation of G6P substrate is overcome and activity is restored to
pre-ischemic levels.

3.3.2 NADP+ phosphatase activity in the isolated rat heart

While NADP+ phosphatase activity has been described in the mitochondria of rat
liver [120], we show here that in the isolated heart if activity exists, it is only in nominal
levels (fig 27). Levels of NADP+ conversion to NAD+, were extremely low, and did not
change after IR; suggesting lysis of the mitochondria as a result of IR does not lead to
release of theoretical NADP+ phosphatase. Therefore, it is unlikely that NADP+
phosphatase is responsible for the depletion of NADP(H) seen in chapter 2.

3.3.3 Location and expression of CD38 during ischemia/reperfusion injury

Immunoblots of hearts subjected to either constant perfusion (control), 30 minutes
of ischemia or 30 minutes of IR showed bands which ran at the same molecular weight as
purified CD38, these bands were not detectable when running western blots with the
blocking peptide (Santa Cruz Biotechnology, Santa Cruz, CA) thus confirming
specificity. Bands of CD38 from heart homogenates showed no difference in expression
across injurious stimuli (fig 28A). Immunohistochemistry of the same isolated hearts
were performed to determine the location of CD38, which appears to be located
predominately within the endothelium of the coronary vasculature and colocalizes with other endothelial enzymes vWF and eNOS (fig 28B).

3.3.4 Effect of ischemia/reperfusion injury on CD38 activity

CD38 activity from homogenates of hearts undergoing 30 minutes of ischemia or 30 minutes IR was measured by production of 2′-P-cADPR. CD38 activity was very low during control, but markedly elevated during ischemia. After 30 minutes of reperfusion activity is 5x that of control levels (fig 31), thus confirming previously published literature which suggests increased CD38 activity during IR injury [121, 143].

3.3.5 Effect of ischemia/reperfusion injury on levels of 2′-P-cADPR

Levels of 2′-P-cADPR, the product of CD38 cyclase activity when using NADP+ as a substrate was measured from isolated hearts subjected to either constant perfusion (control), 30 min ischemia or 30 minutes IR injury (fig 33). Levels of 2′-P-cADPR are nearly undetectable (1.68 nmol/gram tissue ± 0.42), but after 30 minutes of ischemia (10.30 nmol/gram tissue ±0.83; P<0.05 when compared to control) and 30 minutes of IR (10.66 nmol/gram tissue ± 1.04; P<0.05 when compared to control) these levels are greatly elevated (fig 33); corresponding to the in vitro assay of CD38 activity.

3.3.6 Effect of CD38 inhibition on levels of endothelial NADP(H) during ischemia/reperfusion injury
CD38 inhibition was achieved in hearts subjected to IR injury by administering 5mM α-NAD via a sidearm immediately prior to ischemia. Detectable NADP(H) from Triton X-100 treated hearts (as performed in section 2.2.3) was significantly higher when blocking CD38 activity with α-NAD (fig 35).

3.3.7 Effect of CD38 inhibition on eNOS dependent coronary flow during ischemia/reperfusion injury

α-NAD (5mM) delivered just prior to the onset of ischemia (3 min) elevated levels of endothelial NADP(H), this manifested on the whole-heart level as an increase in recovery of eNOS dependent coronary flow upon reperfusion (fig 36). The increase endothelial function would suggest that CD38 activation is detrimental to NOS function post IR injury, and that amelioration of dysfunction can be attributed to the increase in endothelial NADP(H).

3.4 Discussion

In previous reports CD38 was shown to be redox sensitive, where increased exposure to ROS resulted in increased activity of the enzyme [121, 126, 144, 145]. Furthermore, in the setting of IR injury, increased CD38 activity was found, and could be blunted by administration of TEMPO, a free radical scavenger [146]. While literature suggests CD38 activity is increased due to IR, this activity is often measured as a production of cADPR. Here we show for the first time that increased CD38 activity as a result of IR also results in the increase of 2’-P-cADPR, the phosphorylated analogue to
cADPR, which is capable of liberating Ca\textsuperscript{2+} via RyR manner [127, 133]. This new connection between CD38 activation and NOS function is explored in depth below.

3.4.1 Pathway for NADP\textsuperscript{+} reduction is not disturbed

As it was previously eluded to, the modulation of NADP(H) levels depicted in chapter 1 could be the result of either underproduction, overconsumption or the combination of both. For this study we looked at the levels of G6PD activity, the primary synthesizer of NADPH from NADP\textsuperscript{+} in setting of IR in the isolated heart [114]. The results from the study show that G6PD from isolated heart homogenates undergoing various ischemic (30/60 min) and IR (30/60 min) injury was completely normal and did not change across groups (figure 26 left); suggesting the enzyme itself is intact and function despite IR injury.

However, as it was demonstrated with other enzymes, such as eNOS [108] loss of enzymatic function can be achieved through a lack of substrate. Therefore the activity assay was modified so that only endogenous levels of G6P were used (figure 26 right). Interestingly, when using only endogenous levels, the enzymatic activity drops 2-fold during ischemia (both 30 and 60 min). However, regardless of G6P level the enzyme activity is not depressed during reperfusion, the time point when it was found that NADPH and NADP\textsuperscript{+} to be most depleted, thus it is not likely that underproduction of the NADPH is the reason for its decline during reperfusion suggesting overconsumption is the primary mechanism for NADP(H) depletion in IR injury.
3.4.2 Co-localization of eNOS and CD38

As previously discussed above; CD38 has been shown to participate in other phenomena associated with IR injury, and thus its activity could influence NADPH levels (see 3.1.2), however its location within the heart was not previously known. Here we confirm the presence of CD38 within the isolated heart but illustrate for the first time that it co-localizes with von wildebrand factor (vWF) and eNOS within the endothelium of the coronary vasculature (Fig 28B).

This localization to the coronary vessels is intriguing as we have noted thus far that the effect of NADP(H) is most marked within the endothelium. Furthermore, when repletion studies were performed the benefits of adding the eNOS substrate were limited to increases in coronary function but not cardiac function. The expression of CD38 within the coronary arteries vasculature makes it a possible target for mechanistic involvement in NADP(H) depletion.

3.4.3 IR injury increases CD38 activity, and increases levels of the CD38 product 2′-P-cADPR

It has been well documented in literature that CD38 activity is increased as a result of IR injury; however this increase in activity has consistently been measured as either increased catalysis of cADPR or as an increase in CD38 dependent Ca^{2+} release [145, 146]. Whether the aforementioned increase in CD38 activity could catalyze the formation of 2′-P-cADPR (thus limiting NADP^+ availability for NADPH synthesis) has not been shown, and was explored here. The results of these experiments confirm the
increase in CD38 activity due to IR injury (fig 31), however where activity was previously measured as increased cADPR formation, we show that CD38 from isolated hearts undergoing IR injury have the capacity, *in vitro*, to increase synthesis of 2′-P-cADPR. Furthermore, levels of 2′-P-cADPR were found to be elevated in our study in isolated hearts undergoing ischemic or IR injury (fig 33).

3.4.4 Inhibition of CD38 blunts loss of endothelial NADP(H) found as a result of ischemia/reperfusion injury

Thus far we have shown that IR injury results in depleted levels of NADP(H), and this depletion is amplified within the endothelium. Interestingly, when we replete the lost NADPH, only endothelial function in the form of increased coronary flow, but not cardiac function is restored. In section 3.4.2 it was shown that CD38, which has the ability to consume NADP+, was located within the heart and predominately expressed within the coronary vasculature. Furthermore, IR injury increases CD38 capacity to catalyze formation of 2′-P-cADPR, which was found in the whole heart to be elevated in ischemia as well as IR. Thus it is likely that inhibition of CD38 could restore levels of NADP(H) within the endothelium preserving its bioavailability for NOS function and blunt IR induced endothelial dysfunction.

Indeed figure 35 illustrates the restoration of NADP(H) levels to near pre-ischemic values with the addition of α-NAD (5mM), which has been shown previously to elicit inhibitory effects towards CD38 when delivered in high concentrations [147]. Other inhibitors were tested, such as nicotinamide, or nicotinamide mononucleotide (NMN),
however these molecules were unremarkable in their ability to increase endothelial NADP(H). It should be noted that α-NAD (5mM) was unsuccessful at modulating endothelial NADP(H) levels when administered just prior or upon reperfusion, whether this is due to the limitation in efficaciousness of the inhibitor or the result of an ischemic trigger for CD38 which could not be turned off is unknown.

3.4.5 Isolated hearts treated with α-NAD have an increased recovery of eNOS-dependent coronary flow

Inhibition of CD38 returned levels of endothelial NADP(H) to pre-injured values, however the effect on eNOS function remained to be determined. Figure 36 illustrates that the increased levels of NADP(H) demonstrated in figure 35 manifested physiologically as an increase in eNOS dependent coronary flow upon reperfusion. Therefore the activation of CD38 and its subsequent production of 2′-P-cADPR at the expense of NADP⁺, results in restored eNOS function in the wake of IR injury.

Interestingly, the inhibition of NADP(H), while improving coronary flow function did not improve cardiac function. This was seen previously, as NADPH repletion only showed improved effects on coronary flow, but not on cardiac function.

3.5 Conclusion

It must be mentioned that for these studies we did not look at the possibility of poly-ADP-ribose polymerase activity (PARP). PARP is a well known enzyme with an established role in IR injury [111]. While its activity is preferential towards NAD⁺ as a
substrate, it has been previously shown that PARP can utilize NADP$^+$ as a substrate source, albeit poorly [148]. Of importance to PARP activity, when looking to block CD38 activity we did use nicotinamide in high concentrations (5mM). Nicotinamide has been described elsewhere as a viable inhibitor for PARP [149], however in our study, only α-NAD proved to be protective. This may suggest that PARP is not involved, however to definitively make this claim more specific inhibitors such as benzamide [150], 3,4-dihydro5-[4-(1-piperidinyl) butox]-1(2H)-isoquinolinone (DPQ) [150] or aminoisooquinolinone [151] are needed.

IR injury, as previously discussed, brings about endothelial dysfunction, which can limit current therapeutic interventions. While the literature on endothelial dysfunction is vast and our knowledge on how IR injury effects this dysfunction is ever-growing; the ability to mitigate these effects and limit dysfunction still remains elusive. Clinical trials where attempts to bypass endothelial dysfunction and mitigate the no reflow phenomena via NO donors have been conducted, but met with tempered results [152]. The modest results from these trials can be attributed to a multitude of reasons; namely in the oxidative stressed environment of the heart during early reperfusion, addition of exogenous NO may improve coronary flow, but may also increase the production of a powerful oxidant in peroxynitrite. While the goal of these clinical trials and our work is similar, the approach is greatly different. Where the investigators of the clinical trials look to bypass eNOS dysfunction with exogenous NO, we hypothesize that correction of eNOS dysfunction will yield more desirable results than currently reported for clinical trials.
Previously, in chapter 1, we demonstrated that even after 30 minutes of IR injury we were able to ameliorate endothelial dysfunction with the molecule NADPH. Here we offer a new therapeutic target of CD38, which was illustrated to be activated; resulting in the consumption of the NADPH precursor NADP⁺ (fig 37). While high levels of α-NAD were successful in inhibiting CD38 and prevented IR mediated endothelial dysfunction, a more specific inhibitor is critical if these findings are to be translated into therapies.

While we have shown the NADP(H) levels directly influence NOS function in the setting of IR, it is possible that in other diseases aberrations in these levels may indirectly influence NOS function and will be explored in the following chapter.
Figure 23 - The multiple reactions of CD38. CD38 has 4 distinct reactions it catalyzes, (1) formation of the Ca$^{2+}$ liberating molecule cyclic ADP-ribose, in which NAD(P)$^+$ is cyclized. (2) The enzyme also has the ability to hydrolyze cADPR, and forming the molecule ADP-ribose, which can also be done by direct conversion of NAD(P)$^+$ through its (3) glycohydrolase activity. (4) Finally the enzyme, under acidic conditions can produce NAADP$^+$, a molecule which releases Ca$^{2+}$ from lysosomal stores.
Figure 24 - Crystal Structure of CD38. The active site is displayed in purple, which includes the sequence TLEDTLGMY. The crystal structure shows 12 cysteine residues, which are paired. Cysteine pairs which are conserved across the ADP-ribosyl cyclase family are denoted in cyan. One cysteine pair is not conserved and only found in CD38/157 and is colored yellow. Figure is from H.C. Lee: Structure and Enzymatic Functions of Human CD38
Figure 25 - Critical residues of CD38 - Here, only the critical residues for substrate binding are shown. When the substrate is bound the distance between substrate (here depicted as NMN, an analogue of NAD) Asp 155 and Glu 146 is extremely close. It has been postulated that steric hindrance from these residues prevents nicotinic acid from binding at physiological pH. However, under acidic conditions, Asp 155, Glu 146 and nicotinic acid would be protonated, allowing for the base exchange reaction to occur. Also depicted, are the tryptophan residues which can be seen stabilizing the ring of NMN. Figure is from H.C. Lee: Structure and Enzymatic Functions of Human CD38
Glucose-6-Phosphate dehydrogenase isolated from hearts undergoing no injury (PI), varying levels of ischemic injury or varying levels of IR injury, were given NADP⁺ and the conversion to NADPH was followed in a UV-Vis spectrometer at wavelength 260. (Left) Levels of G6PD activity are not significantly different across all groups. (Right) When restricting the assay to use endogenous levels of G6P, activity of G6PD declines by ~50% of PI levels by 30 minutes of ischemia. However, activity returns to pre-ischemic levels during reperfusion. *P<0.05 when compared to pre-ischemic levels.
Figure 27 – NADP\(^+\) phosphatase activity in the isolated rat heart. Homogenates of isolated hearts undergoing constant perfusion (control) or 30 minutes of ischemia/reperfusion (IR) were prepared for NADP\(^+\) activity. Homogenates were first run through a Hi-Trap\(^\text{TM}\) desalting column, then challenged with 20 \(\mu\)M NADP\(^+\). While there is minimal evidence of NAD\(^+\) in control homogenates, this did not change after IR. Therefore, if NADPase does exist within the rat heart, the activity is very low and IR does not increase activity.
Figure 28 - Location of CD38 – Isolated hearts subjected to either constant perfusion (control), 30 minutes of ischemia only or 30 minutes of ischemia plus 30 minutes of reperfusion were prepped for immunoblots or serially sectioned and prepared for immunohistochemistry. A. Immunoblots show a singular band in all groups which ran at the same molecular weight as purified CD38. B. Confocal microscopy of sections (60x) revealed the location of CD38 to be limited predominately to the vessels within the heart. In all sections CD38 colocalized with von wildebrand factor (vWF) and endothelial nitric oxide synthase (eNOS).
Figure 29 – Elution times of various standards. 2'-P-cADPR is not commercially available, therefore the standard was attained by reacting ADP-ribosyl cyclase from *California Apylasia* with NADP⁺ in a Tris-Buffer. This reaction yields the products of 2'-P-cADPR and nicotinamide [127, 140]. Pure nicotinamide and NADP⁺ are also pictured. Peaks were visualized using UV/Vis detection at wavelength 254.
Figure 30 – Representative chromatograms of CD38 activity from homogenates of isolated hearts. Peaks of interest are labeled, and retention times are shown for reference. The first peak which is not labeled overlays the peak from the Tris-buffer when this is run alone. Peaks were detected with UV/Vis at wavelength 254.
Figure 31 - CD38 activity as measured by the production of the molecule 2'-p-eADPR. The enzyme activity was significantly increased 2-fold from non-injured hearts during ischemia (30 min). Activity was further increased with reperfusion where levels are nearly 5x that of non-injured levels. (*P<0.05; **P<0.01 when compared to control levels)
Figure 32 - Representative chromatograms of 2'-P-cADPR in isolated hearts. Chromatograms from isolated heart preps show a common and singular peak which had the same retention time (~6 min) as pure 2'-P-cADPR (observed with UV/VIS at wavelength 254). When pure 2'-P-cADPR was spiked into samples, this overlaid with peaks of interest (denoted by arrow ↓).
Figure 33 – Levels of 2’-P-cADPR. Levels of the CD38 product are elevated during ischemic and IR conditions. Low levels of the calcium modulating molecule 2’-P-cADPR are present in the non-injured isolated hearts. During ischemic (30 min) and ischemia/reperfusion (30 min) injury these levels increase dramatically. 2’-P-cADPR is derived from the metabolism of NADP⁺ via ARC with the additional product of nicotinamide. *P<.05 when compared to control levels.
Figure 34 – Nicotinamide levels during ischemia and reperfusion. CD38 is capable of multiple reactions with different products. One common product in all CD38 reactions is nicotinamide. Increased nicotinamide levels during ischemia are supportive of increased CD38 activity. During reperfusion these levels return back to pre-ischemic levels. Nicotinamide is a membrane permeable molecule, which may explain why these levels are not kept at the elevated levels seen in ischemia. Finally, while nicotinamide levels can be supportive, they are not definitive of CD38 activity as many enzymes produce nicotinamide as a primary or secondary product. *P<0.05 when compared to control levels.
Figure 35 – Effects of CD38 on Endothelial NADP(H). Isolated hearts were either subjected to no injury (control) or 30 minutes of IR injury. An additional group of hearts underwent 30 minutes of IR injury with 5 mM α-NAD infused immediately before ischemia. α-NAD has been shown previously to be an broad ADP-ribosyl cyclase inhibitor, and with its administration resulted in recovery of a majority of endothelial NADP(H). *P<0.05 when compared against IR NADP(H) levels.
Figure 36 – α-NAD effects of Coronary Flow. The broad ADP-ribosyl cyclase inhibitor, α-NAD (5 mM), was given immediately before ischemia. During reperfusion, isolated hearts receiving the CD38 inhibitor consistently showed improved NOS-dependent coronary flow over hearts receiving no intervention. *P<0.01 between treatment groups.
Figure 37 - Mechanism of NADP(H) depletion. 1) BH$_4$ during ischemia is depleted, during reperfusion 2) NADP(H) becomes limiting, and repletion of lost NADPH restores eNOS function. 3) NADP$^+$, the precursor of NADPH is shunted to CD38 which converts NADP$^+$ to 2'-P-cADPR. This conversion of NADP$^+$ to 2'-P-cADPR occurs at a rate 5x that of baseline levels. 4) The shunting of NADP$^+$ to CD38 does not allow for the restoration of NADPH via G6PD.
CHAPTER 4

Importance of NADPH-dependent enzymes in eNOS function

4.1 Introduction

Protein modification is a common occurrence in cells, and often acts as a signaling mechanism, resulting in a change of function, conformation or both. However, not all of these post-translational modifications are beneficial as well as intentional. Of these non-intentional protein modifications glutathionylation of eNOS has been shown to be detrimental in NO synthesis [104]. Proteins can be glutathiolated in the straightforward reaction described below [153]:

\[ \text{PSH} + \text{GSSG} \rightarrow \text{PSSG} + \text{GSH} \]

However levels of the glutathione dimer (GSSG) are extremely low in most tissues, limiting direct protein glutathiolation by GSSG. Furthermore the ratio of reduced to glutathione dimer (GSH/GSSG) is nearly 300-400 times in favor of reduced glutathione levels (GSH).

However, under oxidative stress GSH/GSSG ratio can come closer to balance and increased levels of GSSG favor glutathiolation. Oxidative stress can also prime protein thiols by targeting cysteinyl side chains and generating thiol radicals. Where GSH is free to catalyze the following reaction:
PSH\(^+\) + GSH \(\rightarrow\) PSSG

In elegant work conducted by Chen et al., it was shown that exposing increasing levels of GSSG resulted in a dose dependent cessation of NOS function. Specifically, elevated levels of GSSG drove reactions with cysteine residues (Cys) 689 and 908 within the reductase domain resulting in protein thiol modification. The modification of thiols occurred at the FAD and FMN binding domains, thus disrupting the electron transfer between the flavins as well as increasing their accessibility [104]. The increased access to the surrounding environment allows for \(O_2\) to accept the electron from FAD/FMN, resulting in newly formed \(O_2^-\). This generation of superoxide was only partially inhibited by L-NAME, which quenches \(O_2^-\) generated from uncoupled eNOS from the oxygenase domain, suggesting the generation of free radicals differs from traditional uncoupling [104].

This mechanism on altered enzyme function was demonstrated at the cellular level by inhibiting the NADPH-dependent enzyme GSR, thereby increasing levels of GSSG and promoting NOS glutathionylation. Here, in this chapter, we explore in depth whether these \textit{in vitro} findings are physiologically important and if this new uncoupling mechanism, involving NADPH-dependent enzymes, are found in the pathological state.

4.2 Materials and Methods

4.2.1 Aortic isolation and measurement of relaxation.
Male, Sprague-Dawley rats weighing 275-300 grams were used for these experiments. Rats were given an intraperitoneal (ip) injection of pentobarbital (50mg/kg) and aorta were excised and placed in ice-cold buffer. Adventitia and fat where cleaned from aorta and subsequently cut into rings (5mm in length). Aortic rings were placed in organ chambers and mounted on prongs connected to a force transducer (Multi-Wire Myograph, DMT, Ann Arbor, MI). Organ chambers were filled with Krebs buffer (118 mM NaCl, 18 mM Glucose, 24 mM NaHCO₃, 4.6 mM KCl, 1.2 mM NaH₂PO₄, 1.2 mM CaCl₂, 10 µM indomethacin and 4.6 mM HEPES) warmed to 37°C and bubbled with 95% O₂/5% CO₂. Aortic rings were then allowed to equilibrate for 60 minutes at an initial tension of 1 gram. Stability of rings were checked by administration of 4M KCL. Preps where then washed of KCL (3x, 15 min) with Krebs buffer and allowed to re-equilibrate (15 min) prior to administration of phenylephrine (PHE) (10µM). Once PHE induced stable contraction the endothelial dependent relaxation was measured via a dose response to elevating levels of acetylcholine (ACh) (expressed as the percentage relaxation with respect to the maximal PHE contraction.) To test SMC dependent relaxation a NO-donor was used (NONOate or SNP).

4.2.2 Treatment of isolated aortic segments with 1-3-bis(2-chloroethyl)-1-nitrosourea (BCNU)

Aortic rings were prepared as in 4.2.1, but differed as follows: to induce s-glutathionylation, rings were pretreated with 80 µM 1-3-bis(2-chloroethyl)-1-nitrosourea
(BCNU) during the 60 min equilibration period. Once treated aortic rings were constricted with PHE and underwent a dose-dependent response to ACh.

**4.2.3 Treatment of isolated aortic segments with dithiothreitol (DTT) and BCNU**

Aortic rings were prepared as in 4.2.1, but differed as follows: to reverse S-glutathionylation brought about by exposure of 80 µM BCNU (during 60 min equilibration) 1 mM DTT was added for 20 min (following BCNU exposure). Once treated; aortic rings were constricted with PHE and underwent a dose-dependent response to ACh.

**4.2.4 Treatment of isolated aortic segments from spontaneous hypertensive rats (SHR) with DTT**

Male, spontaneous hypertensive rats (SHR) and wistar-kyoto (WKY; wildtype) rats weighing 275-300 grams were purchased from Charles River and used for these experiments. Rats were given an ip injection of pentobarbital (50mg/kg) and aorta were excised and placed in ice-cold buffer. Aortic rings were prepared for these studies as in section 4.2.1. To reverse the intrinsic S-glutathionylation found in hypertensive rats [104], rings from SHR and WKY rats were treated with 1mM DTT for 20 minutes prior to PHE induced constriction.

**4.2.5 Treatment of SHR with reducing agents**
Male, SHR weighing 275-300 grams were purchased from Charles River and used for these experiments. Rats were given daily IP injections of DTT (17 mg/kg), prior to injections blood pressure (BP) was measured using the non-invasive tail cuff (Columbus Instruments, Columbus, OH). DTT injections and BP measurements were carried out for 7 days. Each session of BP recording included several measurements of BP to ascertain correct levels.

4.2.6 Statistics

Results were expressed as means ± SE. Statistical significance (P < 0.05) was determined by ANOVA (followed by Newman-Keuls test) for multiple groups. Paired or unpaired t-tests were used for comparison between two groups.

4.3 Results

4.3.1 Inhibition of Glutathione Reductase via BCNU results in decreased endothelial

Pretreatment of aortic rings with the GSR inhibitor, BCNU (80µM), resulted in a blunted response to ACh. This response did not improve with increased levels of ACh into the µM range (fig 38). However, when bypassing endothelial function and measuring SMC activity via NO donor, sodium nitroprusside (SNP), this was determined to be normal and did not differ between BCNU-treated and non-treated aortic segments (fig 39; right).
The rationale for using 80 µM BCNU stemmed from previously reported in vitro studies which showed 80 µM BCNU sufficiently blocked glutathione reductase (GSR) and increase levels of GSSG resulting in thiol modification of eNOS [104, 154].

4.3.2 Reversal of glutathionylation in aortic segments

Again, in vitro studies illustrated the ability to remove BCNU induced eNOS dysfunction via the powerful reducing agent DTT [104]. Remaining consistent with in vitro work, 1 mM DTT was added to BCNU treated (as described in 4.2.2) aortic rings. DTT resulted in the restoration of endothelial function by restoring response to ACh across all dosages (fig 40).

4.3.3 Reversal of glutathionylation mediated eNOS dysfunction in aortic segments from SHR rats

eNOS from the aorta of spontaneous hypertensive rats (SHR) has been reported to be glutathionylated [104]. To determine whether this glutathionylation could be removed and restore endothelial dysfunction, aortic rings from SHR were treated with DTT (1mM). Aortic rings derived from SHR without DTT treatment displayed a blunted response to ACh; however when these same aortic rings were treated with DTT (1 mM) the response to ACh was not only restored but enhanced when compared to WKY (control) aortic rings (Fig 41).

4.3.4 Blood pressure does not differ when administering reducing agents in vivo

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Spontaneous hypertensive rats (SHR) from Charles River, were given injections (IP) of DTT (17 mg/kg) everyday for seven days. The effects of DTT on glutathionylated eNOS was measured by a reduction in blood pressure (BP). While DTT was successful in restoring ACh dependent relaxation in the ex vivo aortic ring, this did not translate to the in vivo lowering of BP in SHR. Figure 42 shows a minimal early effect in lowering BP, but this was not determined to be statistically significant (day 3). Regardless, by day 7 systolic and diastolic BP returned to near identical levels as day 1 of treatment.

4.4 Discussion

In the Chen et al. study it is stated that increased oxidative stress could lead to increased levels of GSSG and/or protein thyl radicals resulting in s-glutathionylation. However, the mechanism by which oxidative stress increases GSSG was only partly explored. In chapters 2 and 3 of this thesis work the importance of maintained NADPH levels for eNOS function was demonstrated. Furthermore as explained in section 1.5.1 NADPH is also involved in the re-synthesis of GSH from GSSG. Therefore, it is possible that in the oxidative environment explored by Chen et al. there would be a depletion in the levels of NADPH which would be upstream of increased GSSG and glutathionylation of eNOS. Thus, NADPH could maintain both a direct and indirect influence on eNOS function in the setting of oxidative stress. While the levels of NADPH were not looked at in these studies (explored further in future directions), work from chapters 2 and 3 as well as Chen et al. would suggest depleted NADPH as a potential mechanism.
4.4.1 eNOS dysfunction via inhibition of glutathione reductase in aortic segments

In vitro work by Chen et al. showed the inhibition of glutathione reductase could be achieved through administration of high levels of BCNU (80 µM). In the ex vivo preps using aortic rings from Sprague-Dawley rats, exposure of BCNU in comparable amounts to in vitro assays resulted in decreased sensitivity to ACh. In section 1.2.1 it was discussed how ACh results in increased levels of intracellular calcium which leads to CaM activation; resulting in disassociation of eNOS from caveolin-1. Therefore, a blunted response in the aortic ring to ACh is widely associated with eNOS dysfunction.

BCNU-induced endothelial dysfunction can be overcome by supplementation of NO via sodium nitroprusside (SNP; an NO donor). Response to SNP, again, suggests that the SMC is not affected by BCNU, and suggests that treatment of the GSR inhibitor selectively affects endothelial cells, limiting the production of eNOS dependent NO. In vitro results from Chen et al. would suggest that endothelial dysfunction is brought about by increasing levels of GSSG from blockage of GSR, which leads to glutathionylation of eNOS and uncoupling of the enzyme.

4.4.2 Removal of glutathionylation with powerful reducing agents

To determine if BCNU-induced endothelial dysfunction is reversible we employed the same method used in in vitro studies by Chen et al., where 1 mM DTT effectively removed protein-thiol modifications and restored eNOS function. 1 mM DTT was incubated with aortic rings (which were previously treated with 80 µM BCNU) and subsequently constricted with 10 µM PHE. Figure 40 demonstrates the reversible nature
of glutathionylated eNOS, where 1 mM DTT resulted in the restored sensitivity to ACh. With increasing levels of ACh; relaxation of DTT treated aortic rings, which were exposed to BCNU, resulted in increased relaxation above control (no treatment) aortic rings.

4.4.3 Hypertension as a possible model for glutathionylation related eNOS dysfunction

Figures 38-40 established the ability to induce and reverse endothelial dysfunction whereby aortic rings were exposed to BCNU. However, whether this is a plausible mechanism and explains endothelial dysfunction in pathological states remained to be determined. Chen et al. demonstrated eNOS from aorta of SHR displayed elevated levels of glutathionylation which could be removed when administering DTT (1 mM) [104].

To determine if DTT could restore endothelial function in an ex vivo setting; aortic rings from SHR aorta were treated with 1 mM DTT. Figure 41 illustrated the benefits of DTT treatment, which resulted in increased sensitivity of aortic rings to dose dependent levels of ACh. Relaxation from DTT treated SHR aortic rings was consistently larger than wildtype (WKY) aortic rings. Interestingly, Figure 41 (right) shows DTT treatment not only increased SHR relaxation to ACh but also WKY (wildtype) aortic ring relaxation. This would suggest that there is a basal level of glutathiolated eNOS; indeed this is confirmed in the Chen et al. study where WKY aorta maintained very small but detectable levels of glutathionylated eNOS [104].

4.4.4 Translation into therapy, treatment of blood pressure
While the *ex vivo* aortic ring studies illustrated endothelial dysfunction (SHR or BCNU induced) could be reversed via addition of the powerful reducing agent, DTT, it still remained unknown if DTT could correct pathological states *in vivo*. It was hypothesized that glutathionylated eNOS could be partly responsible for the hypertensive state of SHR and much like the previous *ex vivo* studies this could be reversed with DTT administration. Therefore SHR were administered DTT (17 mg/kg) for one week and blood pressure was measured using a non-invasive tail cuff. Interestingly, the administration of DTT did not result in the decrease of BP over the treatment period. Both systolic and diastolic pressure remained elevated and did not differ significantly from day 1 of treatment to day 7 (fig 42). Why DTT exerted its effect in the *ex vivo* aortic ring but not in the *in vivo* setting of hypertension can be explained by a number of reasons. First IP injection of DTT is not ideal, as we do not know the tissue distribution of DTT, whether it is taken up by and stored in fatty tissue or whether it reaches the endothelium intact to exert its effects. Secondly DTT is known to be toxic, with an LD50 of 400 mg/kg. This became a problem as the days of treatment were extended out to the 5th, 6th and 7th day, where rats became lethargic and demonstrated weight loss, with one rat expiring before the 7th day. Thus, an inherent limitation of DTT is its toxicity, which limited the amount of DTT we could use (17 mg/kg) in the blood pressure experiments. In the aortic ring, this was not a problem and it could be that additional reducing agent was necessary to exert its effects *in vivo*. Due to the conflicting results of the *ex vivo* and *in vivo* work, additional studies will have to be done, where a less toxic reducing agent which is specific for removal of thiol modification can be administered.
4.4.5 Conclusion

Glutathiolation is thought to be a reversible-preventative mechanism, protecting vulnerable cysteines from possible oxidative modification, which can be irreversible. Chen et al. demonstrated that in the pathological state this protective mechanism can become detrimental if prolonged [104]. Here we show that in aortic rings from SHR, the blunted response to ACh can be overcome by administration of DTT. Therefore in the chronic setting of hypertension, elevated GSSG leads to continued glutathionylation of eNOS, which was demonstrated by using the GSR inhibitor BCNU (see 4.3.1). However, if one were to deplete levels of NADPH, this in effect would limit the capacity of GSR to regenerate GSH from GSSG; essentially increasing levels of the glutathione dimer, and lead to glutathionylated eNOS.

While the focus of this work did not directly illustrate an effect of NADPH on eNOS, it did illustrate a mechanism in which NADPH again could be important in maintaining NOS function. While Chen et al. demonstrated glutathionylation of eNOS from SHR, the mechanism for elevated levels of GSSG was only partially explored. It could be, as suggested here, that levels of NADPH become limiting, resulting in the buildup of GSSG and subsequent thiol-modification of eNOS leading to uncoupling.
Figure 38 - Raw data of control and BCNU treated aortic rings. Aortic rings (5mm) where derived from the same aorta and constricted with 10 μM phenylephrine (PHE). Control (no treatment) aortic ring (top curve) sensitivity is evident by its response to the smallest dose of acetylcholine (ACh). BCNU treated aortic rings did not respond to the early-small doses of ACh, but did respond to increased doses of ACh. However, the response; even at elevated levels was blunted when compared to control (no treatment) aortic ring.
Figure 39 - Aortic rings exposed to BCNU displayed a blunted response to acetylcholine (ACh). Control (no treatment) aortic rings maintained a sensitive response to ACh, suggesting intact and functional endothelium. 80µM BCNU was shown in in vitro studies to block glutathione reductase; increasing GSSG levels and leading to glutathionylation of eNOS. The same treatment (80 µM BCNU) as in vitro studies resulted in desensitization of aortic rings to low levels of ACh. Increasing levels of ACh, resulted in relaxation, but this result was blunted when compared to control (non-treated) aortic rings. However when measuring endothelium independent relaxation via addition of the NO donor sodium nitroprusside (SNP); relaxation between non-treated and BCNU treated rings was indistinguishable. Aortic relaxation expressed as % decrease of phenylephrine (PHE)-induced contraction (y-axis); vs. molar concentration of agonist on a logarithmic scale (x-axis). * P<0.05 vs. respective controls.
Figure 40 - Reversible glutathionylation in aortic rings. Endothelial-dependent vasorelaxation was determined in control and BCNU (80 µM) treated aortic rings. BCNU treatment markedly decreased endothelial-dependent maximal relaxation; however sensitivity to ACh was restored when incubating aortic rings with dithiothreitol (DTT) (1mM). Aortic relaxation expressed as % decrease of phenylephrine (PHE)-induced contraction (y-axis); vs. molar concentration of agonist on a logarithmic scale (x-axis). P<0.05 vs. respective controls.
Figure 41 - Endothelial-dependent vasorelaxation was determined in Spontaneous Hypertensive Rats (SHR) and control (WKY) aortic rings. SHR aortic rings displayed a marked decrease in endothelial-dependent maximal relaxation (left panel) to acetylcholine. However, when treating the same SHR rings with 1 mM dithiothreitol (DTT) the endothelial-dependent response to ACh is reestablished. Control (WKY) aortic rings also showed marked increase in response to ACh when pretreated with 1mM DTT (right panel). Aortic relaxation expressed as % decrease of phenylephrine (PHE)-induced contraction (y-axis); vs. molar concentration of agonist on a logarithmic scale (x-axis). P<0.05 vs. respective controls.
Figure 42 - Measurement of blood pressure after treatment with intraperitoneal (IP) injection of DTT. Spontaneous hypertensive rats (SHR) were treated with IP injections of DTT for 7 days and blood pressure was recorded each day. While DTT restored endothelial function in the ex vivo aortic ring this did not translate to the in vivo reduction of blood pressure in SHR rats.
CHAPTER 5
DISCUSSION

5.1 Principle Findings

This section summarizes the most novel findings of chapters 2-4.

1. Ischemia/reperfusion injury results in decreased levels of NADP(H), which disproportionately affects endothelial levels of NADP(H).

2. Repletion of lost NADPH results in restoration of eNOS-dependent coronary flow. The recovery of endothelial function is achieved despite extensive ischemic and reperfusion injury.

3. CD38, which previously has been shown to increase in activity as a result of oxidative stress; here was shown to increase synthesis of 2’-P-cADPR a cyclic molecule derived from the cyclization of the NADPH precursor, NADP⁺.

4. Inhibition of CD38, results in increased levels of NADP(H) within the endothelium of isolated hearts subjected to IR injury. This manifests at the whole heart level as restoration of NOS-dependent coronary flow.

5. Inhibition of the NADPH-dependent enzyme GSR via BCNU, results in decreased endothelial-dependent relaxation in aortic rings.
6. Glutathionylated eNOS found in SHR resulted in decreased endothelial-dependent relaxation in the \textit{ex vivo} aortic rings, but was restored after treatment with DTT.

7. While DTT restored endothelium-dependent vasorelaxation in the \textit{ex vivo} aortic ring, it failed to reduce blood pressure in SHR given IP injections of DTT over several days.
5.2 Clinical implications for NADPH/BH₄ repletion therapy

As discussed in chapter 3, current therapies aimed at eliminating the no reflow phenomenon of IR injury do so by simply bypassing eNOS dysfunction [152, 155, 156]. This is problematic for two reasons: first, in the setting of IR, eNOS is uncoupled, and produces O₂⁻, thus introducing NO via NO donors may lead to formation of OONO⁻ [64]. Second, while NO donor therapy may be successful in increasing coronary flow, this improvement is transient, as NO is quickly metabolized. Therefore it is of no surprise that long-term outcomes as measured by infarct size in these studies was met with minimal beneficial results [152, 155, 156].

What separates the work performed in this thesis and the aforementioned clinical trials; is the goal of our work seeks to restore endothelial dysfunction, not just bypass it. Work by Girlandez et al. would suggest that eNOS is still viable even after severe IR injury; this again is supported by our findings and the findings of Dumitrescu et al. However the results reported by Dumitrescu et al. only demonstrated BH₄ effects on endothelium dysfunction post-IR where recovery was incomplete. In a recent clinical trial reported by Cunnington et al.; the limitations of BH₄ supplementation were highlighted. In the Cunnington study the benefits of oral BH₄ administration prior to CABG surgery was explored [157]. Findings from the Cunnington study showed BH₄ oral supplementation elevated circulating levels of BH₄; however this did not translate to an increase in BH₄ levels within the critical arteries used in bypass surgery. Instead oral BH₄ resulted in increased BH₂ levels within these arteries, which may have been counterproductive [92, 93] and may help explain the deficiency in oral treatment to
increase NO production and/or reduce $O_2^-$ [157]. Interestingly NADPH measurements were not taken during the Cunnington study, but data from our work would suggest that repletion of BH$_4$ levels alone are not adequate to restore NOS function, and would benefit with the repletion of NADPH.

5.3 Endothelial dysfunction and ischemia/reperfusion injury

In chapter 4, we explored the newly detailed mechanism of uncoupled eNOS from glutathionylation of critical Cys residues in the reductase domain; this manifested at the physiological level as a decrease in endothelial specific vasodilation. This response is restored when removing glutathionylation via treatment with DTT, a powerful reducing agent. Furthermore, this newly discovered form of eNOS dysfunction was shown to be a primary contributor to endothelial dysfunction in the animal model of SHR.

A 2003 report released from the National Health and Nutrition Examination Study (NHANES) showed that 53% of participants, who were being treated for hypertension, had a controlled level $\leq$ 140/90 mmHg. [158]. The finding that nearly half of study participants had below optimum control of hypertension is indicative of the prevalent problem of resistant hypertension. Resistant hypertension is classified by the persistent uncontrolled BP in patients after treatment with 3 different classes of antihypertensive drugs (e.g., diuretic, ACE inhibitor, and Ca$^{2+}$ channel blocker).

Combined; the work from chapter 4 and work from Chen et al. illustrates a new therapeutic target for hypertension, a primary risk factor for MI. Therefore it is of interest that patients with uncontrolled hypertension may in fact have glutathionylated eNOS, as
such, current therapies using loop diuretics, ACE inhibitors or Ca\(^{2+}\) channel blockers may not be effective in removing glutathionylation.

5.4 Future Directions

The overall goal of this thesis work was to identify new therapeutic targets which would prevent endothelial dysfunction as a result of pathological disease states (ie IR injury and hypertension). While this study was successful in identifying NADPH as a key modulator of direct and indirect eNOS function in differing disease states, this now becomes a task of translating these findings from the \textit{ex vivo} studies described in this work to therapies which can be used in clinics.

In chapters 2 and 3 we focused on NADPH as an eNOS substrate in IR injury, however much of these studies were performed in the isolated heart limiting the amount of data which can be extrapolated and applied to human disease. With respect to the results of chapter 2; future studies will need to include more \textit{in vivo} data. Specifically, \textit{in vivo} ligation of the left anterior descending artery (LAD) offers a tested and precise model for IR injury, which can be carried out in the rat, thus maintaining species homogeneity with present \textit{ex vivo} studies. An important factor of IR mediated injury includes the immune response. As necrosis of myocardial tissue occurs, this leads to a widespread inflammatory event, which, while necessary to remove necrotic tissues, results in damage to otherwise healthy tissue. In the isolated heart, the inflammatory response is eliminated, however repletion therapy of NADPH/BH\(_4\) may be greatly
influenced by inflammation and therefore its efficacy must be measured in the *in vivo* LAD ligation model.

While the isolated heart model showed near complete recovery of coronary flow, this was not followed by an increase in cardiac function. The reason for this dichotomy in cardiac versus endothelial recovery was discussed in section 2.4.4. It may be that NADPH repletion could be a therapy which can be combined with one that targets specifically the improvement of cardiac function, i.e. stem cells. There exists a myriad of data on stem cell therapy in the setting of IR, where claims of improvement range from increased ejection fraction to nominal reductions in infarct size [159, 160]. A large limitation of stem cell therapy involves the environment in which stem cells are placed [161]. The marginally perfused, highly oxidative environment is not conducive to stem cell survival [162-164]; however in chapter 3 we saw that recovery of coronary flow was eNOS dependent. Therefore, it may be that stem cell therapy with the infusion of NADPH may present a better environment for stem cell survival, thereby increasing cardiac function post-MI.

In chapter 3, we detailed the involvement of CD38 in regulating levels of NADP(H) during IR injury, and that inhibition of this enzyme resulted in improved coronary flow post-ischemia. However future studies need to be undertaken before CD38 inhibition can be used as a viable therapeutic target. While several inhibitors of CD38 exist, the mechanism for these inhibitors is competitive inhibition, therefore in order for these inhibitors to function properly the concentrations are often extraordinarily high (mM range). Thus, future studies must be done with the purified enzyme in order to
develop specific and more efficient inhibitors to CD38. As with the studies with repletion of eNOS cofactors/substrates once a more direct inhibitor of CD38 is produced, it must be tested in the *in vivo* LAD ligation model and its influence on infarct size determined.

Finally, with respect to the clinical applications, the setting of IR injury offers a unique advantage in drug delivery. During percutaneous coronary interventions, catheters are introduced via the brachial, femoral or radial artery to access blockages in the coronary arteries. This direct access to the site of treatment allows for direct drug delivery, and for the purposes of this study would involve the infusion of NADPH/BH$_4$. Another advantage of angiography is the capacity to directly measure changes in coronary flow by determining gradients across coronaries, thus benefits of NADPH infusion could instantly be determined.

In chapter 4 the effects of glutathionylated eNOS was shown to be functionally important to endothelial dependent relaxation in the *ex vivo* aortic ring. Moreover, glutathionylated eNOS, which was shown to be uncoupled, was determined to be pathologically important in the setting of hypertension. While disruption of the NADPH-dependent enzyme GSR was found to be important in these studies and precipitated eNOS glutathionylation, it was not explored if this was occurring during hypertension. In chapters 2 and 3 we saw that NADP(H) levels were modulated during IR injury. It is possible that during hypertension, NADP(H) levels are chronically depleted, which would mirror GSR inhibition; resulting in GSH/GSSG intracellular shift in the ratio towards the glutathione dimer; allowing for eNOS glutathionylation. Future studies should be focused on NADP(H) levels during hypertension, where aorta from SHR and
WKY rats can be prepared for NADP(H) measurements (see 2.2.2) and differences compared.

5.5 Final Remarks

For the first time, in this work, we show the deleterious effects IR injury has on NADPH levels, both at the whole-heart and endothelial level. The depletion of NADP(H) is critical in maintaining eNOS function, as repletion of the enzyme substrate results in a profound recovery of coronary flow. The ability to re-establish coronary flow after such dramatic injury (30 minutes of ischemic injury followed by 30 minutes reperfusion) is of particular importance, as this offers a wide therapeutic window which is advantageous in the clinical setting of IR injury.

Also described in this work is glutathionylated eNOS, which can manifest physiologically as endothelial dysfunction. The finding that DTT treatment of aortic rings from SHR improves endothelium-dependent relaxation brings forward the possibility of a new treatment which may help resolve resistant hypertension. Furthermore, in the aforementioned mechanism the NADPH-dependent enzyme, GSR, was found to be critical in regulating glutathionylation. Thus, results from all studies of this thesis work illustrate that while NADPH has a vast array of important enzymatic influences; it is of particular importance in NOS function and should be explored as a therapeutic target.


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