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Mechanisms of H2O2-induced oxidative stress in endothelial cells

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MECHANISMS OF H_2O_2-INDUCED OXIDATIVE STRESS IN ENDOTHELIAL CELLS

by

Christian Hannon Coyle

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
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Thesis Supervisor: Assistant Professor Khalid N. Kader

ABSTRACT

Development of an *in vitro* model for the early stages of cardiovascular disease is a current necessity. Cardiovascular disease is the leading cause of death in the United States and throughout the world. Oxidative stress and reactive oxygen species have been implicated in cardiovascular disease development. An *in vitro* model of these processes will improve our understanding of cardiovascular disease development and allow for the development of additional treatments.

Atherosclerosis is an inflammatory disease and increased levels of H_2O_2 are associated with inflammation. The model focuses on H_2O_2-induced oxidative stress under static and shear conditions. Previous studies have documented increased $O_2^{\cdot -}$ and increased cytotoxicity in smooth muscle cells exposed to H_2O_2.

Under static culture, endothelial cells exposed to H_2O_2, exhibited increased $O_2^{\cdot -}$ over basal levels via NOS and NAPDH oxidase pathways. Increased $O_2^{\cdot -}$ was attenuated by MnSOD adenoviral-mediated upregulation and endothelial cell exposure to Tiron. This suggests NOS and NADPH oxidase as sources of increased $O_2^{\cdot -}$ under H_2O_2-induced oxidative stress. Endothelial cell cytotoxicity was increased with H_2O_2 exposure. The increase in cytotoxicity was diminished upon exposure to Tiron or L-NAME.

Under shear conditions (8.2 dynes/cm^2), endothelial cells exposed to H_2O_2 exhibited increased $O_2^{\cdot -}$ compared to control via an L-NAME (specific inhibitor NOS) and Apocynin (NADPH oxidase inhibitor) inhibitable mechanism. This suggests NOS and NADPH oxidase as sources of increased $O_2^{\cdot -}$ under H_2O_2-induced oxidative stress. The increased $O_2^{\cdot -}$ was attenuated with MnSOD adenoviral-mediated upregulation and endothelial cell exposure to Tiron (an $O_2^{\cdot -}$scavenger). Endothelial cell attachment under shear with exposure to H_2O_2 was improved with MnSOD adenoviral-mediated upregulation as observed by decreased loss of the endothelial cell monolayer compared with H_2O_2 exposed endothelial cells.

Endothelial cells exposed to H_2O_2 exhibit increased $O_2^{\cdot-}$, suggesting that H_2O_2-induced oxidative stress may be a reasonable model for atherosclerosis. NOS and NADPH oxidase co-inhibition under shear and static culture demonstrated that NOS and NADPH oxidase inhibition is non-additive under static culture, yet additive under shear. Co-inhibition results suggest a complex relationship between the two enzymes that requires additional experimentation to deconvolve.

Abstract Approved: _____
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CERTIFICATE OF APPROVAL

PH.D. THESIS

This is to certify that the Ph.D. thesis of

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To Milton H. Bonney & My Family

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CHAPTER 1
THE CARDIOVASCULAR DISEASE CRISIS

1.1 Introduction

Cardiovascular disease is the leading cause of death in the United States (AHA 2002). Increases in the prevalence of obesity and diabetes mellitus may account for increased deaths due to cardiovascular disease (Frye 2003; Mak et al. 2003). Many current therapies address the immediate life-threatening condition or diseased state, while few implanted devices address the requirements of vascular disease. Pharmacological therapies for the treatment of cardiovascular disease have been successful in clinical trials, notably in the treatment of coronary artery disease (Steinhubl et al. 2001; Kereiakes et al. 2002; Mak et al. 2003). The mechanisms of cardiovascular disease are under investigation. Additional research is needed to further elucidate the underlying mechanisms of atherosclerosis, hypertension, and diabetes mellitus to provide *in vitro* models of the disease states.

Previous studies by Harrison et al. (Cai et al. 2000; Landmesser et al. 2002; Cai et al. 2003b) and Loscalzo et al. (Welch et al. 1997; Maytin et al. 1999; Forgione et al. 2000; Trolliet et al. 2001; Nedeljkovic et al. 2003; Weiss et al. 2003) demonstrate that oxidative stress is an important molecular component of vascular disease, including hypertension and atherosclerosis. Increased understanding of the enzymatic and molecular development of cardiovascular disease may allow for more effective treatment and earlier intervention. This dissertation focuses on the initial development of a model utilizing hydrogen peroxide (H_2O_2)-induced oxidative stress to mimic cardiovascular disease. The model focuses on atherosclerosis, an inflammatory disease (Ross 1999b), associated with increased local levels of H_2O_2 (Halliwell et al. 2000).

The first chapter addresses the current crisis of cardiovascular disease in the United States and world-wide. Cardiovascular disease is not only a grave concern in

industrialized countries. It is also a growing concern in developing nations where prevalence is higher and growing at a faster rate in younger age brackets. This contrasts the current trend in industrialized societies where approximately 50% of those with cardiovascular disease are over the age of 65. The second chapter reviews current literature regarding oxidative stress, reactive oxygen species (ROS), reactive nitrogen species (RNS), and enzymatic pathways that contribute to the production of ROS. This chapter also discusses the relationship between oxidative stress, ROS, RNS, and cardiovascular disease. The third and fourth chapters probe the effects of H_2O_2-induced oxidative stress on endothelial cells under both static and shear culture, focusing on the enzymatic sources of detected ROS and early steps for *in vitro* modeling of cardiovascular disease. The fifth chapter provides a summary of the dissertation and conclusions from the studies.

1.2 Epidemiological Data

Epidemiological data suggests a need for further research into the origins and mechanisms of cardiovascular disease, especially at the cellular and molecular level. All cardiovascular diseases (CVD) combined account for nearly 50% of all deaths in the United States (Figure 1.1). This makes CVD the leading cause of death for both men and women of all ethnicities (AHA 2004a). In addition, increases in ROS have been related to the onset of cardiovascular diseases, including hypertension, atherosclerosis, and diabetes mellitus (White et al. 1994; Mugge 1998; Kojda et al. 1999; Pepine et al. 2001; Harrison et al. 2003a). Our understanding of the early stages and mechanisms of cardiovascular disease is still developing. The development of a model for cardiovascular disease and oxidative stress is a necessity to improve and further our understanding of cardiovascular disease at the cellular level.

1.3 Mortality and Morbidity

Nearly 930,000 Americans died from cardiovascular diseases (CVD) in 2001, with coronary heart disease (CHD) accounting for approximately 54% of those deaths

(AHA 2004a). This is significant especially considering the prevalence of cardiovascular disease in the United States, which was reported to be 22.6% (2001). More than 1 out of every 5 Americans suffers some form of CVD (AHA 2004a). Further, other diseased states may also be present with CHD, including hypertension and diabetes mellitus. Of the nearly 64.5 million Americans suffering from CVD, more then 50 million have hypertension as defined by the 2001 standard (blood pressure above 140/90, (AHA 2004a).

These statistics suggest that a large majority of patients suffering from cardiovascular disease are likely to have chronic oxidative stress, which is thought to be associated with their diagnosed disease state (Harrison et al. 2003a; Prasad et al. 2003; Vassalle et al. 2004). Oxidative stress and ROS are also thought to be important in the progression of atherosclerosis, due to their role in endothelial cell gene expression (Eyries et al. 2004). This exemplifies the need for continuing study and modeling of cardiovascular disease states, especially as the prevalence continues to rise. The need to study cardiovascular diseases is further supported with the increasing trends of obesity in both adults and children and an increasing prevalence of type II diabetes in children, both major risk factors for cardiovascular disease (AHA 2004a; AHA 2004b).

Clearly the crisis is already severe with more then 50% of deaths due to cardiovascular disease (AHA 2004a), but there is evidence to suggest that increased prevalence is likely in the future if current trends persist. Additional development of vascular disease is also likely as a consequence of increasing life-spans in the United States. More then 80% of Americans over the age of 75 are expected to have some form of cardiovascular disease. The epidemic of cardiovascular disease encompasses industrialized and non-industrialized countries, more then one-third of world wide deaths are due to cardiovascular disease (AHA 2004b). The highest rates of growth of cardiovascular disease diagnosis are in poorer nations where the prevalence of disease is

significantly higher in young age brackets, compared to higher prevalence in older age brackets in industrialized nations.

1.4 Economic Impact

Significant resources are currently allocated to health care and the treatment of cardiovascular disease. Direct and indirect costs for cardiovascular disease were estimated at nearly 337 billion dollars for 2004 (AHA 2004a). As a comparison, the cost for cardiovascular disease is nearly 15% of the total yearly federal budget. At a time when health care costs are increasing significantly and benefits are stagnant or decreasing, the issue of cost has become a significant political and societal issue. Early intervention would be ideal, and the use of H_2O_2 is proposed to attempt to provide insight into the mechanisms of cardiovascular disease development with elevated H_2O_2. In the future, it is hoped that this research will be utilized to develop a model for cardiovascular disease and aid in the search for new therapeutic opportunities to allow for earlier intervention.

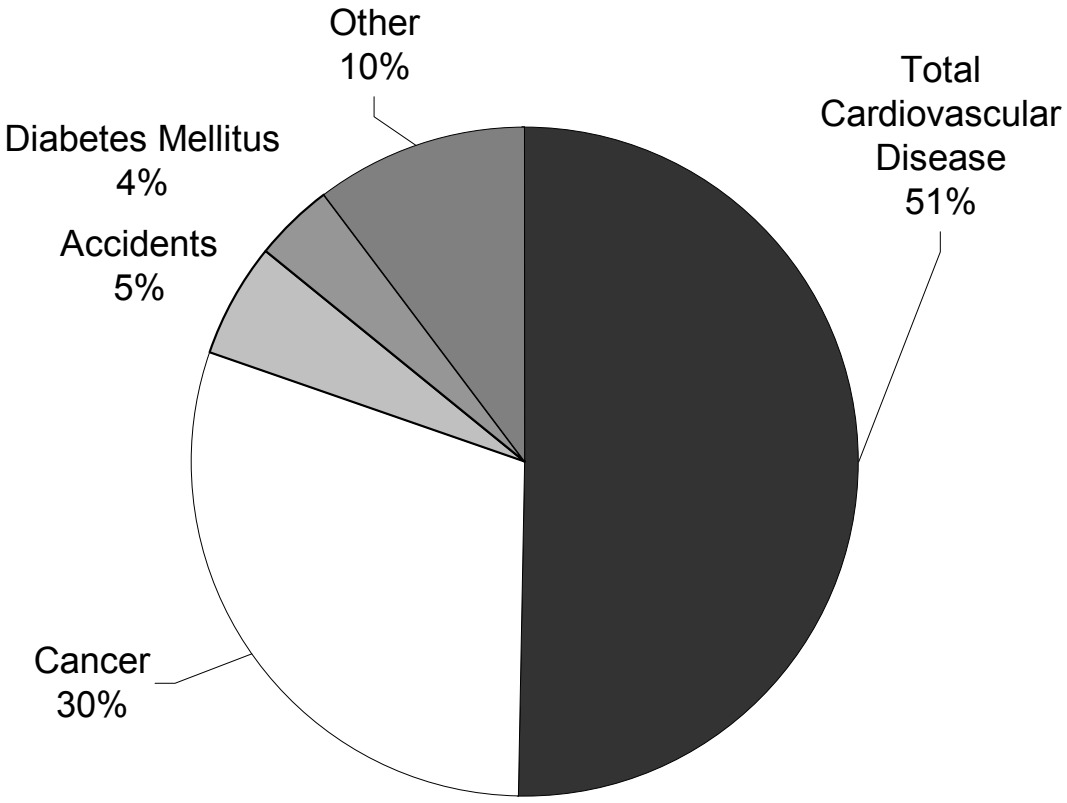

Figure 1.1: Total Cardiovascular Disease is a Significant Cause of Death in the United States. This figure provides a graphical representation of the distribution of deaths in the United States (AHA 2004a). Nearly 51% of deaths were due to cardiovascular disease. An additional 4% of deaths were due to diabetes mellitus, which is associated with vascular disease. Cardiovascular disease is the primary cause of death in the United States.

CHAPTER 2
AN INTRODUCTION TO OXIDANT SPECIES, OXIDANT ENZYMATIC SOURCES, AND OXIDANT STRESS

2.1 Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) have multiple roles within the cardiovascular system. At lower concentrations, ROS and RNS are important signaling molecules. ROS, including H_2O_2, also participate in pathway signaling related to cellular proliferation, migration, and apoptosis (Brown et al. 1999; Rhee 1999; Griendling et al. 2000c; Patel et al. 2000a). At higher concentrations, ROS and RNS participate in the alteration of cellular phenotype from a basal state to an activated state resulting in increased inflammatory signaling and in increased ROS and RNS formation. The increase in ROS, RNS, and inflammatory signaling results in increased leukocyte and platelet activation and increased leukocyte recruitment (Patel et al. 2000b; Cooper et al. 2002; Stokes et al. 2002a; Stokes et al. 2002b). The modification of cellular phenotype and increased levels of ROS and RNS are associated with oxidative stress and vascular disease formation and progression.

Increased pro-oxidants are associated with vascular disease and are thought to be an important early step in vascular disease development, including atherosclerosis and hypertension (Harrison et al. 2003a; Landmesser et al. 2003; Vassalle et al. 2004). Multiple pro-oxidants and anti-oxidants participate in the normal physiologic balance that is lost with oxidative stress.

2.2 Cardiovascular Disease and Oxidative Stress

ROS and oxidative stress are implicated in atherosclerosis, hypertension, and diabetes development (Giugliano et al. 1995; Griendling et al. 2003; Hwang et al. 2003; von Baeyer et al. 2003). The superoxide anion ($O_2^{\cdot-}$) is thought to have a major role in

the development of atherosclerosis (Hink et al. 2001) and hypertension (Li et al. 1997; Hink et al. 2001). Endothelial cells have been shown to exhibit increased $O_2^{\cdot-}$ in response to unregulated glucose (Shinozaki et al. 2001) and during endothelial cell dysfunction (Fenster et al. 2003).

In early atherosclerotic development, it has been proposed that endothelial cells contribute to ROS levels and ROS formation (Cai et al. 2003b; Landmesser et al. 2003), while smooth muscle cells and fibroblasts are induced to form ROS after endothelial cell dysfunction (Harrison et al. 2003c; Landmesser et al. 2003). Previous studies have demonstrated increased vascular smooth muscle cell $O_2^{\cdot-}$ levels with H_2O_2 exposure (Li et al. 2000). In addition, increases in systemic plasma H_2O_2 levels have been observed in patients with cardiovascular disease (Lacy et al. 1998). Oxidative stress and increased ROS are associated with multiple cardiovascular disease states.

2.2.1 Atherosclerosis

Atherosclerosis has been documented as an inflammatory disease and is associated with increased $O_2^{\cdot-}$ and other ROS (Griendling et al. 2000b; Patel et al. 2000a; Patel et al. 2000b; Mertens et al. 2001; Harrison et al. 2003b; Lassegue et al. 2003; Wassmann et al. 2004). Possible signaling mechanisms for the inflammatory based reaction are discussed in section 2.3.

An early focus in atherosclerosis has been oxidized low density lipoprotein (LDL), one of the major components of atherosclerotic lesions (Cucina et al. 1998; Ross 1999c; Ross 1999a; Chisolm et al. 2000; Morawietz et al. 2001; Li et al. 2003; Shatrov et al. 2003; Zettler et al. 2003). Endothelial cell dependent relaxation of vascular smooth muscle cells (SMC) is impaired in atherosclerosis (Cominacini et al. 2001) due to decreased availability of nitric oxide (NO). Reduction in NO availability may result in an additional down-regulation of NO formation, though this is still controversial (Ignarro 1990). Decreased NO availability may also be linked with increased $O_2^{\cdot-}$ due to NO scavenging by $O_2^{\cdot-}$.

Additional studies have demonstrated NADPH oxidase is in turn activated by oxidized LDL (ox-LDL) and angiotensin II in smooth muscle cells, resulting in increased output of $O_2^{\cdot-}$ by NADPH oxidase (Yokoyama et al. 2000). Increased $O_2^{\cdot-}$ output via NADPH oxidase also results in elevated levels of H_2O_2 allowing for an additional increase in multiple pro-oxidants derived from H_2O_2 and $O_2^{\cdot-}$.

Byproducts of lipoxygenases, such as the HPODE family, have also been shown to lead to increased $O_2^{\cdot-}$ in both endothelial cells and smooth muscle cells (Li et al. 2003). There appears to be a complex interaction between pro-oxidants and anti-oxidants in normal cellular function and cardiovascular disease (Figure 2.1).

2.2.2 Hypertension

Abnormal hemodynamic flow disturbances are more likely to be generated in a hypertensive cardiovascular system compared with a non-hypertensive system, as observed in essential hypertension (Jones et al. 1990) and with atherosclerosis lesion development (Stehbens 1982; Davies et al. 2001). The modulation of shear stress, abnormal flow patterns, and altered shear stress may lead to cellular damage. Patients with hypertension often develop other cardiovascular diseases, most notably atherosclerosis (Landmesser et al. 2002). Endothelial cell dysfunction is often associated with hypertension, due to elevated levels of shear stress. Higher levels of shear stress have been shown to damage the endothelium resulting in increased ROS formation, including $O_2^{\cdot-}$ (Zalba et al. 2001).

Various reports have suggested that oxidative stress plays a critical role in hypertension (Munzel et al. 1999; Cai et al. 2000; Griendling et al. 2000b; Lassegue et al. 2003; Wassmann et al. 2004). As in atherosclerosis, NO availability is reduced, possibly contributing to the structural remodeling observed in hypertensive vessels. There are two major markers of hypertension, endothelial cell dysfunction and vascular smooth muscle cell hypertrophy. These changes are due to an increase in oxidative stress and linked with decreased NO availability (Zalba et al. 2000). The reduction in nitric oxide

availability is tied to increased $O_2^{\cdot-}$ levels, mainly contributed by NADPH oxidase in smooth muscle cells, resulting in NO intereaction with $O_2^{\cdot-}$ and the formation of peroxynitrite ($ONOO^-$, (Landmesser et al. 2002; Rathaus et al. 2002). Loss of the NO-$O_2^{\cdot-}$ balance results in cellular damage, contributing to hypertension and the loss of endothelial cell control of vessel dilation (Zalba et al. 2001). Angiotensin II also stimulates NADPH oxidase formation of $O_2^{\cdot-}$, resulting in dysfunctional endothelial cells as discussed above (Hanna et al. 2002; Landmesser et al. 2002; Rocic et al. 2003).

2.2.3 Diabetes Mellitus

Oxidative stress and the resulting endothelial cell dysfunction is also associated with diabetes mellitus, where NO availability is again reduced in part through NOS uncoupling (Caimi et al. 2003). NOS uncoupling results in endothelial cell dysfunction and formation of $ONOO^-$ due to the interaction of NO and $O_2^{\cdot-}$ and oxidation of the NOS cofactor BH_4 (Channon et al. 2002; Caimi et al. 2003). Diabetic patients often develop other vascular diseases, including atherosclerosis and hypertension. Accelerated progression of atherosclerosis is associated with diabetes mellitus (Watson et al. 2003). NOS uncoupling could possibly be due to glucose stimulation of NOS and increased NO formation, in parallel with endothelial cell dysfunction. This scenario would result in increased $O_2^{\cdot-}$ formation allowing for increased formation of $ONOO^-$ (Heitzer et al. 2001; Hink et al. 2001).

2.3 Inflammation

Inflammation has been implicated in cardiovascular disease, notably in atherosclerosis (Liuzzo 2001; Eldika et al. 2004) and essential hypertension (Kristal et al. 1998). Inflammation is associated with a shift in endothelial cell phenotype, resulting in increased expression of inflammatory mediators, cytokines, and iNOS activation (Granger et al. 2004). H_2O_2 participates in the regulation of multiple inflammatory pathways including VCAM-1 expression, ICAM-1 expression, NF-kB translocation, and PECAM-1 expression through a multi-step signaling process. In atherosclerosis, the

inflammatory activity of H_2O_2 attracts and increases the likelihood of monocyte and macrophage adhesion to the endothelial cell surface, contributing to chronic inflammation (Granger et al. 2004).

H_2O_2 is initially formed from $O_2^{\cdot-}$ produced by NADPH oxidase during inflammation, possibly through angiotensin II activation of NADPH oxidase (Pueyo et al. 2000; Cai et al. 2003b; Granger et al. 2004). Angiotensin II can also stimulate NF-kB translocation to the nucleus resulting in VCAM-1 expression (Pueyo et al. 2000) and inflammation. In a complimentary pathway, TNF-A and PKC also contribute to the formation of ROS, again resulting in NF-kB activation as well as ICAM-1 expression (Lakshminarayanan et al. 1997; True et al. 2000; Ahmad et al. 2002). In a review by Granger et al., angiotensin II and NADPH oxidase were implicated in ROS formation resulting in the expression of VCAM-1, PECAM-1, ICAM-1, E-selectin, and P-selectin, all of which promote leukocyte adhesion (Granger et al. 2004). Additional implicated factors include Fe^{2+} (Chen et al. 2004), MCP-1 gene expression (Chen et al. 2004), and RAC-1 (Lakshminarayanan et al. 1997; Ahmad et al. 2002; Chen et al. 2004) in the process of inflammation via H_2O_2 stimulation. H_2O_2 plays an important role in inflammatory signaling.

2.4 Oxidative Stress

Oxidative stress is associated with cardiovascular disease including atherosclerosis, hypertension, and diabetes mellitus (Giugliano et al. 1995; Griendling et al. 2003; Hwang et al. 2003; von Baeyer et al. 2003). Oxidative stress is described as an imbalance between anti-oxidant and pro-oxidant species or as the deregulation of pro-oxidants and anti-oxidants. The deregulation concept addresses the variable roles played by $O_2^{\cdot-}$ and H_2O_2 in vascular cells in both the endothelial cell basal state and the diseased state.

$O_2^{\cdot-}$ and H_2O_2 play a role in normal cellular function and cellular signaling. Vascular cells maintain anti-oxidant mechanisms to reduce the impact of acute oxidative

stress, scavenge ROS produced during normal cellular function and respiration, and to regulate aspects of cellular signaling via $O_2^{\cdot-}$ and H_2O_2. Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), the hydroxyl radical (OH·), superoxide anion ($O_2^{\cdot-}$), and the RNS, peroxynitrite ($ONOO^-$) (Griendling et al. 2000b; Griendling et al. 2000c; Cai et al. 2003a; Taniyama et al. 2003) contribute to the pro-oxidant/anti-oxidant imbalance (Figure 2.2). In a non-diseased cardiovascular system, $O_2^{\cdot-}$, NO, and other pro-oxidants and anti-oxidants are regulated. With onset of cardiovascular disease, the pro-oxidant/anti-oxidant balance is lost and pro-oxidants increase with an eventual decrease in anti-oxidant availability and antioxidant enzyme expression during chronic oxidative stress(Honing et al. 1998; Hamilton et al. 2004; Muller et al. 2004), notably the RNS nitric oxide (NO). The oxidant imbalance is normally associated with increased $O_2^{\cdot-}$ and decreased NO availability (Iuchi et al. 2003; Zhang et al. 2003).

$O_2^{\cdot-}$ is an important ROS in the vasculature as it is pivotal in generating other ROS and RNS species including H_2O_2 (Hassan et al. 1981; Fridovich 1983), OH· (Yang et al. 1995; Wolin 2000), $ONOO^-$ (Davidson et al. 1997; Wink et al. 1998; Wolin et al. 1998) and HOCl (Taniyama et al. 2003). NO at elevated concentrations, also plays a role in the formation of additional RNS species, including NO_2^- (Wolin 2000).

While ROS and RNS are considered to be toxic, there is significant evidence to suggest that they are utilized in cellular signaling. This is especially true of H_2O_2, which has been implicated in multiple signaling cascades (Griendling et al. 2000a; Cai et al. 2003a), notably angiotensin II signaling in smooth muscle cells and inflammation (Harrison 1997; Griendling et al. 2000c).

2.4.1 Pro-oxidants

ROS and RNS are major pro-oxidants found within the vasculature. $O_2^{\cdot-}$, H_2O_2, and ONOO- are three of the key pro-oxidants and will be discussed in greater detail. Additional pro-oxidants include OH·, HOCl, and lipid radicals, which have all been shown to contribute to oxidative stress (Wassmann et al. 2004).

2.4.1.1 Superoxide Anion

$O_2^{\cdot-}$ is primarily formed through a one electron reduction of O_2 (Wolin et al. 2002). It is produced by various oxidases and enzymes within endothelial cells, smooth muscle cells, and fibroblasts, discussed in section 2.4.2. $O_2^{\cdot-}$ is also essential as it can form additional ROS and RNS including H_2O_2 (interaction between 2 $O_2^{\cdot-}$) and $ONOO^-$ (interaction between NO and $O_2^{\cdot-}$, (Radi et al. 1991; Hanna et al. 2002; Landmesser et al. 2003).

$O_2^{\cdot-}$ is also utilized for intracellular signaling including activation of the ras/rac-Raf1-MAPK pathway (Brandes 2003; Buetler et al. 2004) and angiotensin II signaling in vascular cells (Griendling et al. 2000b). $O_2^{\cdot-}$ also stimulates the release of intracellular Fe^{2+} stores (Wolin 2000), allowing for the formation of OH· through the reaction of H_2O_2 with Fe^{2+} and other free metal ions (Cu^+, Wolin et al. 2002; Brandes 2003). In addition, $O_2^{\cdot-}$ participates in cell proliferation, migration, and apoptosis regulation through its ability to form H_2O_2 (Brown et al. 1999; Rhee 1999; Wolin et al. 2002). To reduce $O_2^{\cdot-}$, it is converted to H_2O_2 by superoxide dismutase (SOD), discussed in section 2.4.3, and through $O_2^{\cdot-}$ self-interaction.

2.4.1.2 Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is a stable ROS, an important signaling molecule in vascular cells, and is capable of diffusing through cellular membranes (Ohno et al. 1985). In the normal vasculature, H_2O_2 formation is primarily reliant upon $O_2^{\cdot-}$ production and interaction/dismutation of $O_2^{\cdot-}$ to form H_2O_2, allowing for cellular signaling (Rhee et al. 2000; Lassegue et al. 2003).

Studies over the past 8 years have demonstrated the importance of H_2O_2 to intracellular signaling mechanisms. H_2O_2 is an important signaling molecule in vascular cell apoptosis (Brown et al. 1999), proliferation (Brown et al. 1999), and modulation of intracellular Ca^{2+} levels (Dreher et al. 1995). It also upregulates eNOS gene expression and activity, and increases NO production (Cai et al. 2003b). In addition, H_2O_2 is

required for PDGF, EGF, and angiotensin II signaling (Sundaresan et al. 1995; Bae et al. 1997; Ushio-Fukai et al. 1999).

H_2O_2 also mediates the activation and response of important protein kinases including MAP kinase (Machino et al. 2003; Blanc et al. 2004), tyrosine kinase (Aslan et al. 2003; Frank et al. 2003a; Konishi et al. 2004), Src (Seshiah et al. 2002; Wang et al. 2004), and PKC (Niwa et al. 2002; Frank et al. 2003b; Wang et al. 2004) in vascular cells. H_2O_2 also appears to play a more general role in the phosphorylation and dephosphorylation of serine groups, resulting in modification of protein activity. One example is eNOS, where H_2O_2 stimulates eNOS upregulation and activity resulting in increased NO production (Cai et al. 2003b). Previous studies by Harrison et al. have demonstrated eNOS upregulation with H_2O_2 exposure via a calcium dependent mechanism (Drummond et al. 2000; Cai et al. 2001). A recent study also suggests that H_2O_2 may contribute to insulin resistance through its inhibition of the Akt pathway in rat aortic smooth muscle cells (Yamaguchi et al. 2003).

In disease states, H_2O_2 is utilized in the formation of additional ROS including $OH\cdot$, and HOCl (Taniyama et al. 2003). H_2O_2 is degraded into H_2O via catalase and glutathione peroxidase (Cai et al. 2003a), discussed in section 2.4.3.

2.4.1.3 Peroxynitrite

Peroxynitrite ($ONOO^-$) is a potent and damaging pro-oxidant in the vasculature. $ONOO^-$ is formed through the interaction of NO and $O_2^{\cdot-}$ (Squadrito et al. 1995; Wattanapitayakul et al. 2000; Guzik et al. 2002) with a reaction rate three times the rate of SOD reaction with $O_2^{\cdot-}\cdot$ (Darley-Usmar et al. 1995; Faraci et al. 2004). The reaction to form $ONOO^-$ is dependent upon equimolar concentrations of NO and $O_2^{\cdot-}$. SOD scavenging of $O_2^{\cdot-}$ is the dominant reaction until the intracellular concentrations of NO and $O_2^{\cdot-}$ are roughly equivalent (Faraci et al. 2004). NO availability is reduced with elevated concentrations of $O_2^{\cdot-}$, through the formation of $ONOO^-$.

$ONOO^-$ has multiple effects on cellular signaling including inhibition of PGI_2 synthase (Zou et al. 1999), upregulation of PGH_2 via stimulation of cyclooxygenase (Wolin et al. 1990), lipid peroxidation (Ronson et al. 1999; Radi et al. 2001), NOS decoupling (Kuzkaya et al. 2003; Landmesser et al. 2003), DNA oxidation and damage (Ronson et al. 1999; Radi et al. 2001), cell damage and protein oxidation, (Ronson et al. 1999; Radi et al. 2001), inhibition of mitochondrial respiration (Levonen et al. 2001; Radi et al. 2002), and an overall reduction in intracellular nitric oxide availability (Liu et al. 1998; Kuzkaya et al. 2003). Oxidation of proteins, DNA, and lipids are associated with increased levels of $ONOO^-$, though $ONOO^-$ oxidative action is reversible unlike that of OH· (O'Connor et al. 1997; McConnell et al. 2003; Virag et al. 2003).

2.4.1.4 Additional Pro-Oxidants

Two additional pro-oxidants are HOCl and OH·. HOCl is formed through myloperoxidase modification of H_2O_2 and NO_2^- (Wassmann et al. 2004). HOCl may also contribute to the oxidation of low density lipoprotein (LDL, (Podrez et al. 2000).

OH· is formed through Fenton chemistry via H_2O_2 intereaction with available metal ions, such as Fe^{2+} and Cu^+ (Winterbourn 1995; Tsou et al. 1996). OH· is highly reactive and damages non-specifically and irreversibly at its site of formation. The formation of OH· results in oxidation of proteins, DNA, and lipids as well as endothelial cell dysfunction and eventual cell death (Kvietys et al. 1989; Beckman et al. 1990; Visseren et al. 2002).

2.4.2 Sources of Reactive Oxygen Species

Multiple sources of reactive oxygen species (ROS) have been identified in vascular cells. These include: xanthine oxidase (Miyachi et al. 1986; Satoh et al. 1998; Fleming et al. 2001), NADPH oxidase (Meyer et al. 1999; Hohler et al. 2000; Barry-Lane et al. 2001; Beswick et al. 2001; Seno et al. 2001; Brandes et al. 2002; Li et al. 2002; Lassegue et al. 2003; Parinandi et al. 2003), and uncoupled NOS (Beretta et al. 2003; Landmesser et al. 2003). NADPH oxidase and uncoupled NOS are thought to be major

contributors to intracellular $O_2^{\cdot-}$. Additional ROS sources include the mitochondrial electron transport chain (Poderoso et al. 1998b), lipoxygenase (Kukreja et al. 1986; Giardina et al. 1998), glucose oxidase (al-Bekairi et al. 1994), and cytochrome P450 (Bai et al. 2001; Fleming et al. 2001). The primary ROS produced by these oxidases is $O_2^{\cdot-}$, except for xanthine oxidase which can produce both $O_2^{\cdot-}$and H_2O_2, and glucose oxidase which directly produces H_2O_2 (Figure 2.3).

Another possible pathway for $O_2^{\cdot-}$ formation in endothelial cells is uncoupled NOS. The likelihood of NOS uncoupling varies with the cellular environment. Low or oxidized tetrahydrobiopterin (BH_4) and/or low concentrations of L-arginine are associated with NOS decoupling (Ishii et al. 1997; Milstien et al. 1999; Kuzkaya et al. 2003; Ulker et al. 2003). Previous reports demonstrate that exposure of vascular smooth muscle cells to H_2O_2 results in increased $O_2^{\cdot-}$ (Li et al. 2000). In addition, NOS contributes to endothelial cell $O_2^{\cdot-}$ formation upon exposure to native LDL (nLDL) (Stepp et al. 2002).

2.4.2.1 NADPH oxidase

A non-phagocytic NADPH oxidase has been identified in vascular cells (Griendling et al. 1994; Mohazzab et al. 1994a; Mohazzab et al. 1994b). The components of the NADPH oxidase protein complex vary by vascular cell type (Griendling et al. 2000b; Lassegue et al. 2001). NADPH oxidase is membrane bound and forms $O_2^{\cdot-}$ using either NADPH or NADH and O_2 (Munzel et al. 1999; Griendling et al. 2000b). The oxidase consists of multiple proteins that form a complex upon activation, proteins may include $p47^{phox}$, NOX proteins, $p67^{phox}$, and $p22^{phox}$ (Figure 2.4). NADPH oxidase is activated via multiple signaling cascades including angiotensin II (Hanna et al. 2002). Activation of NADPH oxidase results in c-Src activation, stimulation of EGFR, PI3K, translocation/modification of Rac-1, and then phosphorylation and translocation of $p47^{phox}$ (Cai et al. 2003a). Translocation of $p47^{phox}$ results in NADPH oxidase formation

of $O_2^{\cdot-}$ and eventual H_2O_2 formation resulting in a feed forward mechanism with H_2O_2 stimulation of c-Src (Figure 2.5).

Munzel et al. demonstrated significant $O_2^{\cdot-}$ production through NADPH oxidase in both smooth muscle cells and endothelial cells under basal conditions (Munzel et al. 1999). NADPH oxidase also contributes to $O_2^{\cdot-}$ levels in vascular cells with the onset of cardiovascular disease, including atherosclerosis, hypertension, and diabetes mellitus related cardiovascular complications (Griendling et al. 2000b; Sorescu et al. 2001; Cai et al. 2002).

2.4.2.2 Uncoupled NOS

There are two primary isoforms of NOS present in endothelial cells, endothelial NOS (eNOS) and inducible NOS (iNOS). In addition, there is the more controversial mitochondrial NOS (mtNOS). eNOS is the primary producer of NO in endothelial cells and is a Ca^{2+} dependent isoform (Alderton et al. 2001). eNOS consists of two domains, including the reductase domain and the oxygenase domain (Figure 2.6). iNOS is expressed in endothelial cells via cytokine production and inflammatory signaling, it is not Ca^{2+} dependent (Alderton et al. 2001; Aktan 2004). Each isoform is reliant upon several cofactors for proper function, including tetrahydrobiopterin (BH_4), FAD, FMN, and iron bound to the heme site. NOS also requires NADPH, O_2, and L-arginine to produce NO (Alderton et al. 2001).

Inadequate levels of co-factors and L-arginine can result in NOS dysfunction and uncoupling, altering NOS output from NO to $O_2^{\cdot-}$ (Vasquez-Vivar et al. 1998; Huang et al. 2000). Dysfunction of NOS also can occur with oxidation of the heme complex or BH_4 by $ONOO^-$ also resulting in uncoupled NOS (Figure 2.7, (Huang et al. 2000; Harrison et al. 2003b). Uncoupled NOS may contribute to the pathogenesis of vascular disease.

Uncoupled NOS has been implicated in $O_2^{\cdot-}$ formation due to endothelial cell exposure LDL. NADPH oxidase, NOS to a lesser extent, and xanthine oxidase are

implicated in increased ROS in endothelial cells upon exposure to minimally oxidized LDL (oxLDL, (Heinloth et al. 2000; Cominacini et al. 2001; Rueckschloss et al. 2001; Stepp et al. 2002). NADPH oxidase is also an important contributor to $O_2^{\cdot-}$ levels in smooth muscle cells due to H_2O_2 exposure (Li et al. 2001).

2.4.2.3 Xanthine oxidase

Xanthine oxidase is a potent source of $O_2^{\cdot-}$, can also produce H_2O_2 directly, and is present in endothelial cells and at greater concentrations in circulation. Xanthine oxidase is converted from xanthine dehydrogenase (Granger 1988) and can then form $O_2^{\cdot-}$ and H_2O_2 from hypoxanthine, xanthine, and NADH (Wolin 2000). It also appears to have an important role in ROS production during ischemia, reperfusion, and with high levels of LDL (Granger 1988; Ohara et al. 1993; Granger 1999). This suggests xanthine oxidase as a likely contributor to ROS in atherosclerosis. With formation of H_2O_2, there is the possibility that the OH· radical will be formed through the reaction of H_2O_2 with available metal ions (Winterbourn 1995; Tsou et al. 1996).

2.4.2.5 Cyclooxygenase

Cyclooxygenase is also a source of $O_2^{\cdot-}$ within endothelial cells. It plays a central role in the arachadonic acid pathway and PGH_2 production. PGH_2 is an important precursor for PGI_2 released by endothelial cells to modulate platelet activity and smooth muscle cell contractility (Kukreja et al. 1986; Holland et al. 1990). Cyclooxygenase produces $O_2^{\cdot-}$ through its ability to oxidize NADPH and alternative substances to NADP·, which autoxidizes O_2 resulting in $O_2^{\cdot-}$ (Kukreja et al. 1986). Cyclooxygenase is also capable of generating significant quantities of ROS while producing prostaglandins such as PGH_2 (Holland et al. 1990; Marshall et al. 1990).

2.4.2.6 Lipoxygenase

Lipoxygenase has been shown to produce $O_2^{\cdot-}$ and is also associated with the arachidonic acid cascade (Parthasarathy et al. 1989). It may play a role in the oxidation of LDL and formation of lipid free radicals, which are capable of scavenging NO

(Parthasarathy et al. 1989; Mertens et al. 2001). Lipoxygenase is also thought to contribute to angiotensin II activation of NADPH oxidase $O_2^{\cdot-}$ formation during cardiovascular disease (Luchtefeld et al. 2003).

2.4.2.7 Mitochondrial Respiration

The overall significance of mitochondrial ROS production and its importance in cardiovascular disease have not been significantly explored. The mitochondrial electron transport chain produces $O_2^{\cdot-}$ from two sites, the co-enzyme Q region and NADH dehydrogenase (Boveris 1977; Poderoso et al. 1998a). Several studies have suggested a role for mitochondrial production of $O_2^{\cdot-}$ in stimulation of vascular cell apoptosis (von Harsdorf et al. 1999; Cadenas et al. 2000; Panaretakis et al. 2001). Mitochondrial respiration can be inhibited by both $ONOO^-$ and NO. With NO inhibition, an increase in ROS production by mitochondria has been observed (Brunori et al. 1999).

2.4.2.8 Myeloperoxidase and Iron

Myeloperoxidase is an important source of ROS in the circulation and can result in more powerful radical formation including OH·, the most damaging ROS. Myeloperoxidase directly forms HOCl (Carr et al. 2000a) a strong acid used alongside H_2O_2 and OH· to degrade invaders in the body as part of the immune system response (including macrophages, (Carr et al. 2000b; Wassmann et al. 2004). HOCl is formed via myloperoxidase in circulation and vascular cells and is associated with macrophage oxidation of LDL (Eiserich et al. 2002). Myeloperoxidase is also heavily expressed in macrophages and other immune cells (Carr et al. 2000b).

Iron (Fe^{2+}) is an essential component for cellular function. Within an oxidative environment, Fe^{2+} release contributes to the formation of OH· (Kvietys et al. 1989; Visseren et al. 2002). Intracellular Fe^{2+} stores can be released by $O_2^{\cdot-}$ and NO stimulation (Beckman et al. 1990; Davidson et al. 1997; Alderton et al. 2001), this likely plays a role in OH· formation with increased metal ion availability during oxidative stress.

2.4.3 Anti-oxidants

Superoxide dismutase (SOD), catalase, and glutathione peroxidase are the primary anti-oxidants that will be discussed. Anti-oxidant systems provide for the regulation of pro-oxidants in cellular signaling and also balance the level of pro-oxidants during acute oxidative stress. There are additional non-enzymatic anti-oxidants, including vitamins C and E. Vitamin C is taken up by endothelial cells and stored as ascorbate to provide for $O_2^{\cdot-}$ scavenging within the cell (Wassmann et al. 2004).

Higher concentrations of anti-oxidants and pro-oxidants have also been shown to inhibit and reduce the activity and expression of anti-oxidants, notably MnSOD (MacMillan-Crow et al. 1999). This is of importance during vascular disease where pro-oxidant regulation is lost and cells are exposed to chronic oxidative stress.

2.4.3.1 Nitric Oxide

Nitric oxide (NO) is not an anti-oxidant as it does not actively neutralize pro-oxidants, though it can react with pro-oxidants and is a RNS. With normal cellular function, eNOS is the primary source of NO in endothelial cells (Alderton et al. 2001; Landmesser et al. 2003). Proper production of NO is associated with normal endothelial cell function, vasorelaxation, and maintenance of a basal endothelial cell phenotype (Alderton et al. 2001; Landmesser et al. 2003). The basal phenotype includes proper regulation of smooth muscle cells (Harrison et al. 2003b), of the coagulation cascade, of platelet activity (Alderton et al. 2001; Harrison et al. 2003b), of non-inflammatory signaling, and maintenance of normal cellular signaling. NO availability appears to be one of the primary factors in vascular disease development. Reduced NO levels and availability is highly correlated with vascular disease (Patel et al. 2000a; Stokes et al. 2002b; Harrison et al. 2003b; Kaysen et al. 2004).

Excess levels of NO have been shown to be cytotoxic. This is observed primarily in inflammation with activation of iNOS and increased iNOS NO production. iNOS is activated during inflammatory signaling and produces NO at high rates (Brunori et al.

1999). This allows cells to be exposed to cytotoxic levels of NO. NF-kB trans-location to the nucleus also increases the activity of iNOS, resulting in increased NO availability and increased formation of $ONOO^-$ or NO cytotoxicity with disease (Aktan 2004; George et al. 2004).

In cardiovascular disease, NO availability is primarily reduced through scavenging by pro-oxidant species, including $O_2^{\cdot-}$, lipid free radicals, and nitryl free radicals. With chronic oxidative stress, NO availability may further decrease, through a reduction in eNOS expression and activity (Vaziri et al. 2001; Harrison et al. 2003b; Pandolfi et al. 2003). NO is thought to be key in delaying the onset of cardiovascular disease as lower levels of NO are associated with most cardiovascular disease states, though this is still controversial (Wink et al. 1998; Wolin 2000).

NO has also been shown to inhibit mitochondrial respiration where inhibition results in increased ROS output from mitochondria (Radi et al. 2002). In contrast, $ONOO^-$ inhibition of mitochondrial respiration has not been shown to increase $O_2^{\cdot-}$ output from mitochondria.

2.4.3.2 Superoxide Dismutase

There are three isoforms of superoxide dismutase (SOD), Cu/ZnSOD localized in the cytosol, MnSOD localized in the mitochondria, and ecSOD localized in the extracellular spaces between endothelial and vascular smooth muscle cells. ecSOD also associates with heparan sulfate proteoglycan on the endothelial cell membrane after production by SMC (Faraci et al. 2004). SOD is the primary scavenger of $O_2^{\cdot-}$ in endothelial cells, through a reduction of two $O_2^{\cdot-}$ molecules resulting in H_2O_2 and O_2 (Griendling et al. 2000a; Salvemini et al. 2002). The total percentage of each SOD isoform expressed in different vascular cells was recently reviewed by Faraci et al. (Faraci et al. 2004). High levels of MnSOD were found in endothelial cells.

Previous studies have demonstrated a reduction in LDL oxidation by endothelial cells and reduced endothelial cell $O_2^{\cdot-}$ levels with adenoviral mediated upregulation of

Cu/ZnSOD and MnSOD (Fang et al. 1998). An additional study has shown that upregulation of Cu/ZnSOD mediates the shear stress suppressive effects of apoptosis in endothelial cells (Dimmeler et al. 1999). $ONOO^-$ at higher concentrations has been demonstrated to inhibit MnSOD expression and transcription (MacMillan-Crow et al. 1999).

2.4.3.3 Catalase and Glutathione Peroxidase

Catalase is localized in intracellular peroxisomes and in the cytosol (Wassmann et al. 2004), where it reduces H_2O_2 to H_2O and O_2 through a two-step reaction. Catalase is thought to be important in severe oxidative stress by reducing intracellular H_2O_2, which is the byproduct of $O_2^{\cdot-}$ self-interaction and SOD (Faraci et al. 2004).

Glutathione peroxidase is thought to play a significant role in the oxidant balance in vascular cells. It reduces H_2O_2 and lipid peroxidases to H_2O and lipid alcohols (Wassmann et al. 2004). Inadequate expression or dysfunction of glutathione peroxidase can contribute to $OH\cdot$ formation by not detoxifying H_2O_2. At lower levels of expression, glutathione peroxidase also may allow for lipid peroxidase reaction with free metals to form lipid peroxyl radicals (Wolin et al. 2002; Wassmann et al. 2004).

2.4.3.4 Vitamin C & Vitamin E

Vitamin C scavenges ROS directly and does not alter the activity of ROS producing enzymes (Gotoh et al. 1992; Fontana et al. 1999). Vitamin C is also thought to salvage oxidized BH_4 by regenerating BH_3^-, the intermediate step of BH_4 oxidation, to BH_2 by $ONOO^-$ (Baker et al. 2001; Katusic 2001; Toth et al. 2002; d'Uscio et al. 2003). Vitamin E is lipid soluble, scavenges ROS directly, and may have a role in protecting against LDL oxidation (Fontana et al. 1999; Carr et al. 2000c; Shimazu et al. 2001).

2.5 Shear Environment

In several recent studies, altered endothelial cell phenotype and morphological changes were observed due to endothelial cell exposure to different shearing conditions, including laminar flow (Davis et al. 2001), steady flow, oscillatory flow (Hwang et al.

2003), pulsatile flow (Silacci et al. 2000; Silacci et al. 2001), and disturbed flow (Ziegler et al. 1998). Pulsatile flow is the natural flow state in the vasculature (Milnor 1972), while oscillatory flow may be found in smaller arteries and capillaries (Lew 1972).

Alterations in endothelial cell behavior under different flow conditions has previously been demonstrated (Malek et al. 1995). Endothelial cells are stimulated by shear, resulting in increased NOS expression (Tsao et al. 1996; Ziegler et al. 1998; Kader et al. 2000) and SOD expression (Takeshita et al. 2000). An increase in NO levels was also observed with exercise (Ennezat et al. 2001; Wung et al. 2001; Davis et al. 2003) suggesting further increases in NO production under shear.

2.5.1 Gene Expression

Previous studies have examined endothelial function under uni-directional shear using a cone and plate technique (Hwang et al. 2003; McNally et al. 2003). These studies found endothelial cell phenotype and function varied between static and shear conditions (Wasserman et al. 2004). One notable change was an increase in NO formation (Buga et al. 1993) and eNOS mRNA upregulation (Mattart et al. 2003), which could alter the cellular environment and affect the pro-/anti-oxidant balance. Endothelin and the endothelin converting enzyme mRNA expression have also been shown to be downregulated under shear (Masatsugu et al. 2003; Mattart et al. 2003).

Endothelial cell function and gene expression is also altered under different shear conditions, including laminar flow and oscillatory flow (Malek et al. 1995; Ando et al. 1996; Boyle et al. 1997; Takahashi et al. 1997; Traub et al. 1998; Sorop et al. 2003; Uhlenbrock et al. 2003). Altered endothelial cell function is important in vascular disease states, as atherosclerotic lesions tend to form in regions with low shear stress or regions of oscillatory shear (De Keulenaer et al. 1998; De Nigris et al. 2001; Harrison et al. 2003a).

Genetic studies have documented upregulation of cycloxygenase-2, NOS, and MnSOD under laminar shear (Dimmeler et al. 1999; Ennezat et al. 2001). This suggests

increases in anti-oxidant protection under laminar shear and protection from apoptosis as no increase in gene expression was observed under non-laminar shear conditions (Topper et al. 1996). It is suggested that gene expression in endothelial cells under different forms of shear will contribute to the likelihood of atherosclerotic development based upon regulation of anti-oxidant and pro-oxidant signaling mechanisms (Brooks et al. 2004; Wasserman et al. 2004).

2.5.2 Pro-oxidant and Anti-oxidant Formation

Under laminar shear conditions, decreased $O_2^{\cdot-}$ levels and monocyte adhesion have been observed (Berk et al. 2002; Hwang et al. 2003). An additional study has suggested that laminar shear increases ICAM-1 expression and leukocyte adhesion (Chiu et al. 1997), this was shown for a short time course. In contrast, the study by Hwang et al. suggested an initial increase (short-time course) with a fall-off to basal levels after several hours of laminar shear exposure. Laminar shear has also been shown to increase CuZnSOD and NO production mediating endothelial cell apoptotic signaling under shear (Dimmeler et al. 1999). Increases in NOS activity and NO formation via increased intracellular Ca^{2+} (Manevich et al. 2001), increased formation of PGI_2 (Wang et al. 1997), and increased glutathione activity (Takeshita et al. 2000) have all been attributed to laminar shear stimulation.

Under oscillatory shear, different cellular behavior was observed compared to laminar shear conditions. eNOS expression was increased via H_2O_2 signaling (Cai et al. 2004) and there was also an increase in $O_2^{\cdot-}$ levels (Silacci et al. 2001; Hwang et al. 2003). Increased NOS expression and activity allows for a possible increase in $ONOO^-$ formation under oscillatory shear. This phenomenon may contribute to a possible feed forward mechanism with angiotensin II activation and long-term $O_2^{\cdot-}$ production (Kuzkaya et al. 2003). Along with increases in $O_2^{\cdot-}$, increased leukocyte and monocyte adhesion were observed under oscillatory flow (Yeh et al. 2001; Berk et al. 2002; Hwang et al. 2003; McNally et al. 2003). Increased leukocyte adhesion and activation may relate

to the formation of atherosclerosis as it tends to be located in sections of vessels with oscillatory shear or regions of low shear stress (Wang et al. 1997; Silacci et al. 2001).

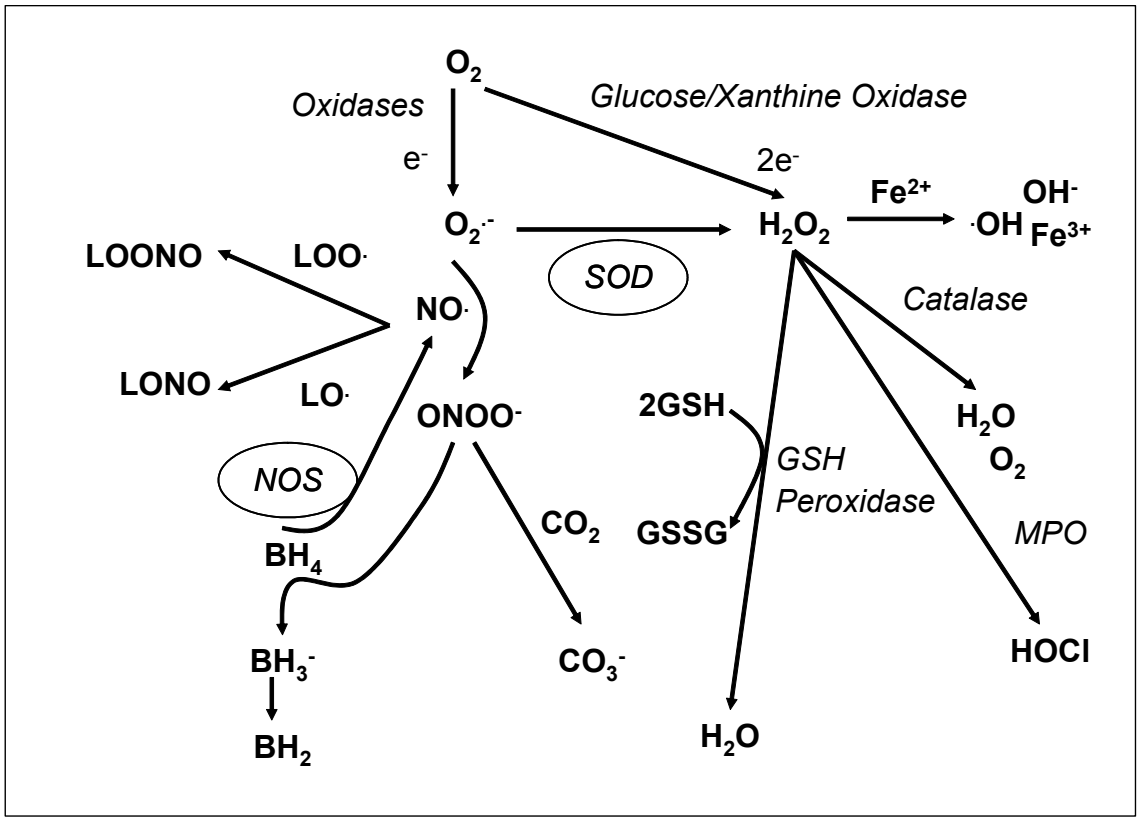

Figure 2.1: Pro-oxidant and Anti-oxidant Interactions. This figure depicts the complex interactions that may begin with formation of superoxide from different vascular cell oxidases (Figure 2.3). It includes the formation of lipid free radicals ($LOO^{\cdot}$) and their scavenging of NO as well as NOS decoupling, SOD scavenging of $O_2^{\cdot-}$, and the formation of $OH^{\cdot}$. It also depicts the reactions for removal of H_2O_2 and the corresponding degradation products.

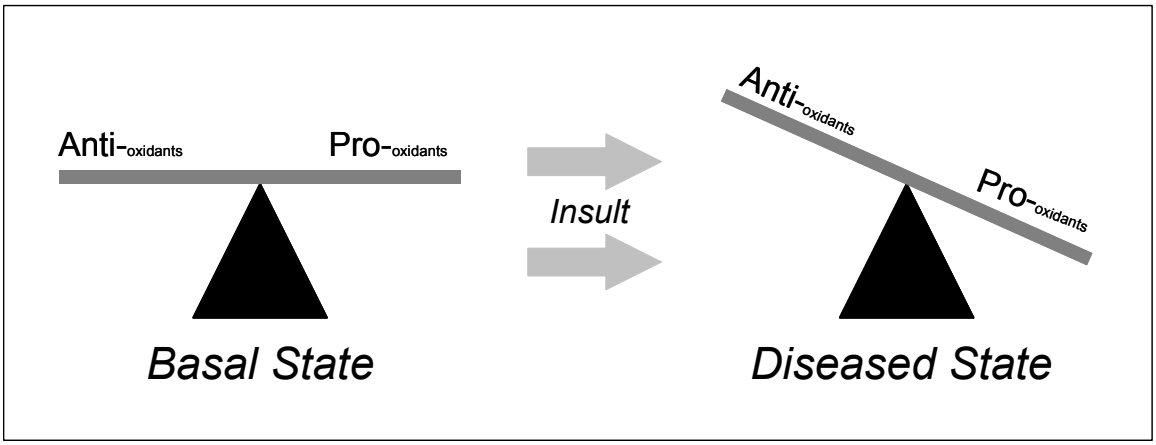

Figure 2.2: Oxidant Balance in Basal and Disease States. This figure visualizes the balance observed between anti-oxidants and pro-oxidants in a basal state. With an insult or endothelial cell dysfunction the balance is lost resulting in an increase in pro-oxidants overwhelming available anti-oxidants.

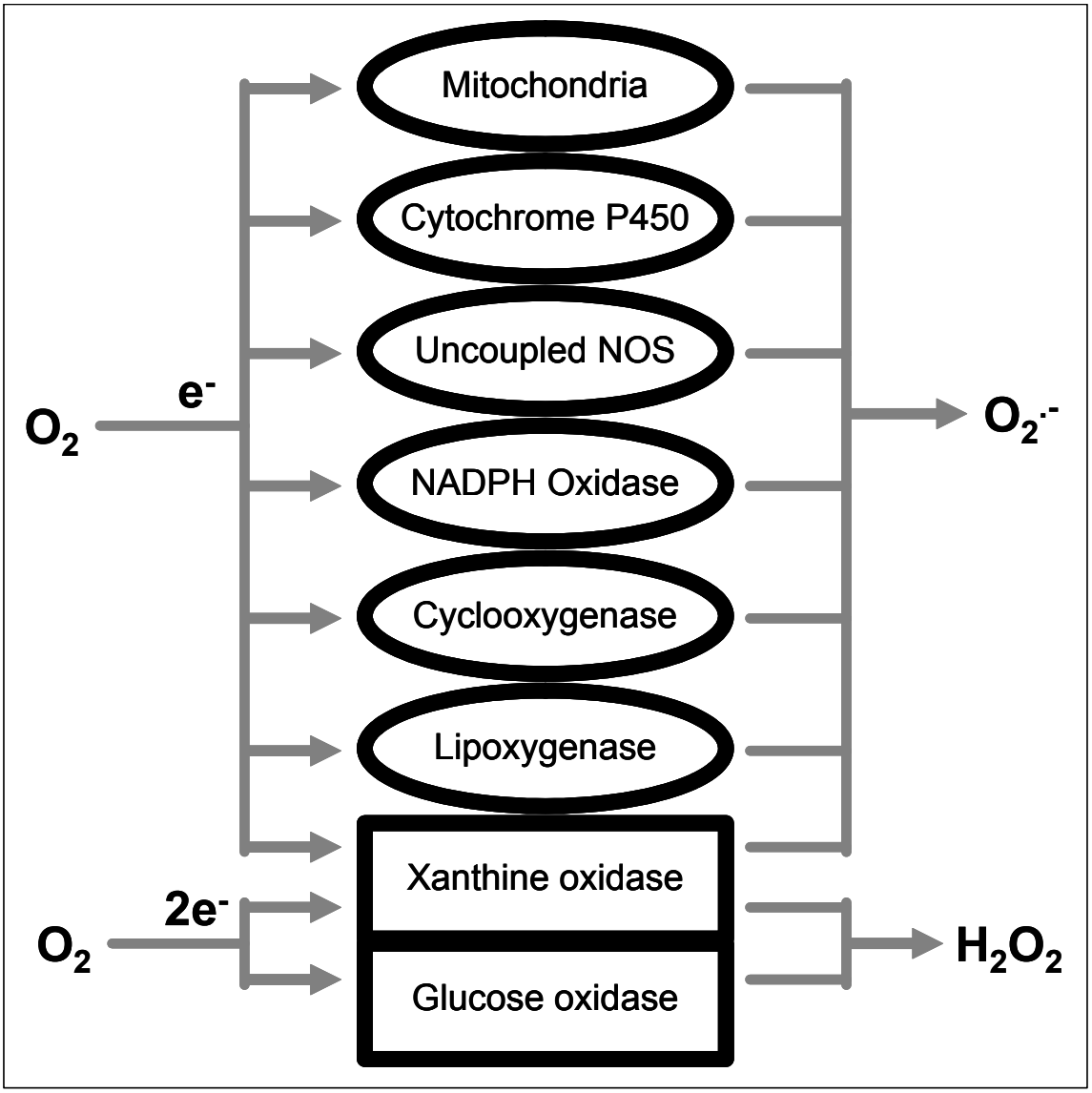

Figure 2.3: Enzymatic Sources of $O_2^{\cdot-}$ and H_2O_2. This figure demonstrates sources of superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) in vascular cells.

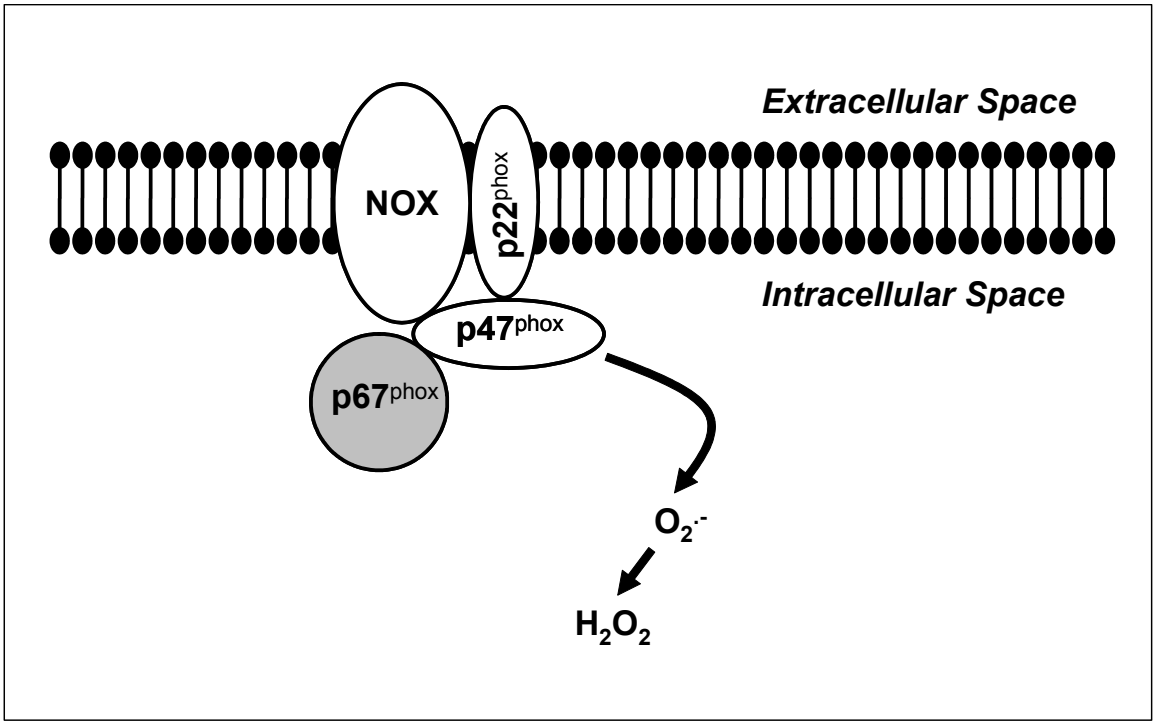

Figure 2.4: NADPH Oxidase Structure. This figure depicts the generalized proteins associated with the NADPH oxidase complex. The formation of the protein complex including phosphorylation and translocation of $p47^{phox}$ are necessary for $O_2^{\cdot -}$ production via NADPH oxidase.

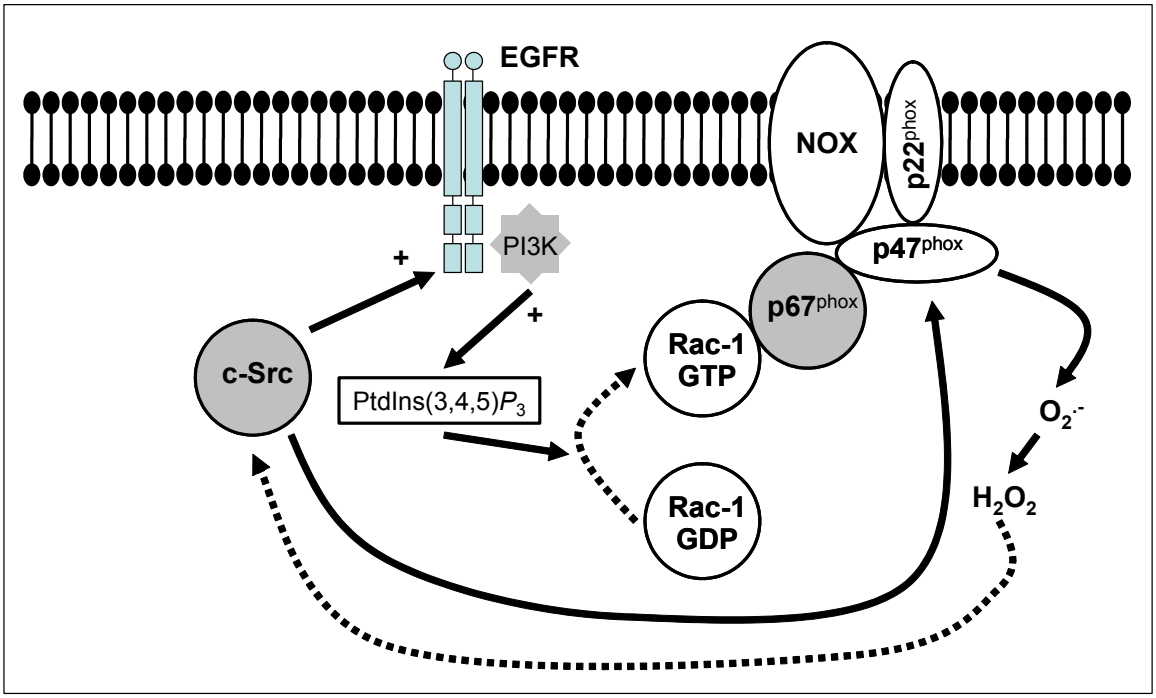

Figure 2.5: NADPH Oxidase Activation. This figure visualizes one of the activation cascades for NADPH oxidase formation of $O_2^{\cdot-}$. This involves activation of c-Src and its phosphorylation of $p47^{phox}$ and the translocation of $p47^{phox}$ resulting in $O_2^{\cdot-}$ formation. c-Src interaction with the EGFR receptor and translocation of Rac-1 results in further increases in NADPH oxidase production of $O_2^{\cdot-}$. H_2O_2 can be formed from $O_2^{\cdot-}$ and act as positive feedback stimulating c-SRC resulting in further increases in $O_2^{\cdot-}$ production.

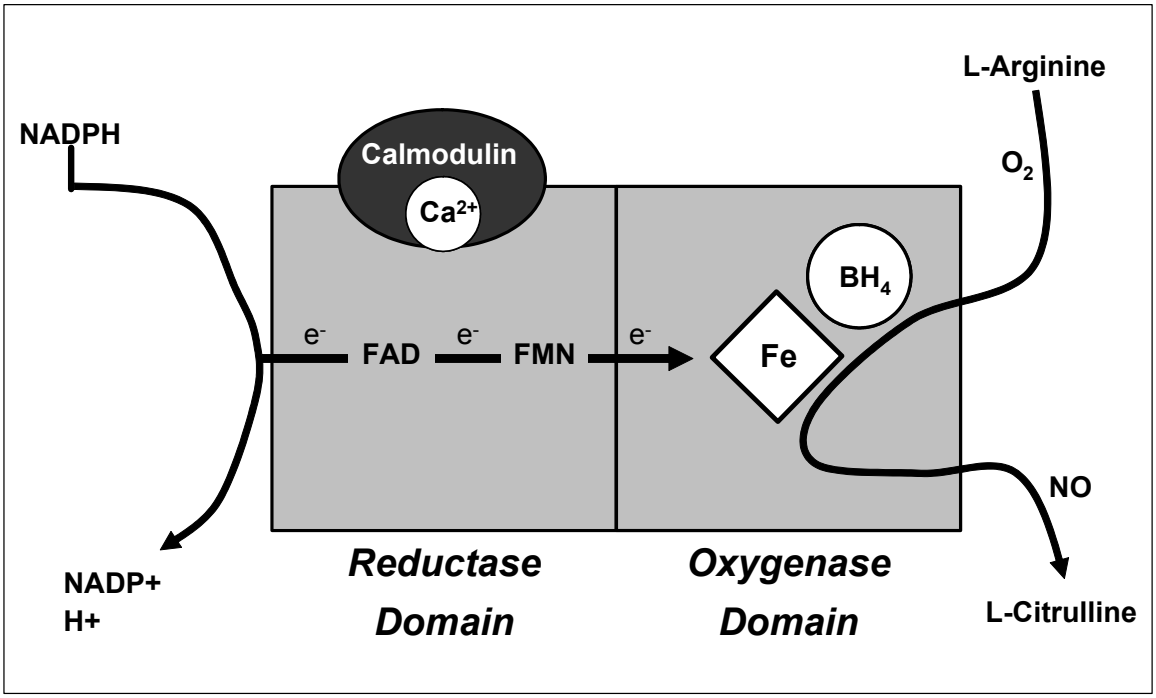

Figure 2.6: NOS Structure. This figure visualizes the structure and location of co-factors on the two domains of eNOS. The reductase domain is where Calmodulin (Ca^{2+}), FAD, FMN, and NADPH interaction occurs and is part of the electron transport chain in eNOS. The oxygenase domain is the location of the heme site (Fe) and BH_4 and is where L-Arginine and O_2 are utilized to form NO.

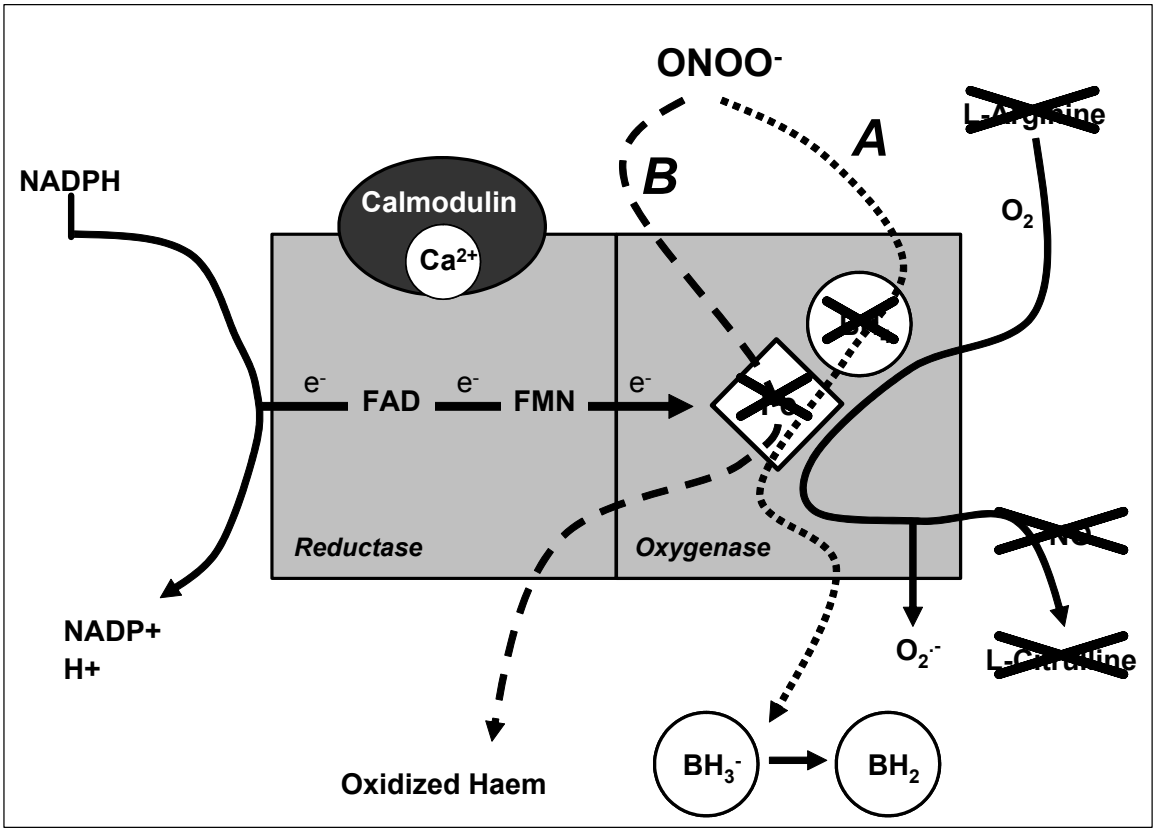

Figure 2.7: Uncoupled NOS. This figure depicts the uncoupling of NOS, where $ONOO^-$ oxidizes the heme structure (B) or BH_4 (A) resulting in $O_2^{\cdot-}$ formation instead of NO formation.

CHAPTER 3
MECHANISMS OF H_2O_2-INDUCED OXIDATIVE STRESS IN ENDOTHELIAL CELLS

3.1 Abstract

Hydrogen peroxide is produced by inflammatory and vascular cells and induces oxidative stress that may contribute to atherosclerosis and endothelial dysfunction. In smooth muscle cells, H_2O_2 induces production of $O_2^{\cdot -}$ by activating NADPH oxidase. However, the mechanisms whereby H_2O_2 induces oxidative stress in endothelial cells are poorly understood. Here, we examined the effects of H_2O_2 on $O_2^{\cdot -}$ levels on porcine aortic endothelial cells (PAEC) under static culture and laminar oscillatory shear. Treatment with 60 μmol/L H_2O_2 markedly increased intracellular $O_2^{\cdot -}$ levels (determined by conversion of dihydroethidium to ethidium bromide) and produced cytotoxicity (determined by propidium iodide staining) in PAEC under static culture conditions. Overexpression of human manganese superoxide dismutase in PAEC by adenoviral-mediated gene transfer reduced $O_2^{\cdot -}$ levels and attenuated cytotoxicity resulting from treatment with H_2O_2. L-NAME, an inhibitor of nitric oxide synthase (NOS), and apocynin, an inhibitor of NADPH oxidase, reduced $O_2^{\cdot -}$ levels in PAEC treated with H_2O_2, suggesting that both NOS and NADPH oxidase contribute to H_2O_2-induced $O_2^{\cdot -}$ in PAEC. To assess responses to H_2O_2 in PAEC under shearing conditions, a flow rate of 24.4 ml/min was applied to produce physiologically relevant shear stress (8.2 dynes/cm^2). As was observed under static conditions, treatment with H_2O_2 increased $O_2^{\cdot -}$ levels in PAEC exposed to laminar shear stress. We conclude that H_2O_2 produces oxidative stress in endothelial cells by increasing intracellular $O_2^{\cdot -}$ levels through NOS and NADPH oxidase. These findings suggest a complex interaction between H_2O_2 and oxidant-generating enzymes that may contribute to endothelial dysfunction in atherosclerosis.

3.2 Introduction

Oxidative stress causes endothelial dysfunction and cellular injury, which contribute to atherosclerosis (Harrison et al. 2003a) and other cardiovascular diseases (Cai et al. 2000). $O_2^{\cdot -}$ is produced by a variety of cellular enzymes, including NADPH oxidase, xanthine oxidase, cyclooxygenase, cytochrome P450, and mitochondrial respiratory chain enzymes (Cai et al. 2000; Wolin et al. 2002; Cai et al. 2003a). In addition, endothelial nitric oxide synthase (NOS) can produce large amounts of $O_2^{\cdot -}$ when the enzyme becomes uncoupled from its normal substrates (Milstien et al. 1999; Witteveen et al. 1999; Vasquez-Vivar et al. 2002; Zou et al. 2002; Landmesser et al. 2003). $O_2^{\cdot -}$ is converted to H_2O_2 spontaneously or through the action of superoxide dismutase. $O_2^{\cdot -}$, H_2O_2 and their reaction products modulate numerous aspects of vascular cell function.

H_2O_2 in the plasma is kept at low levels because of reactions with heme proteins, sulfhydryl groups, and ascorbate, suggesting that vascular endothelial cells encounter little circulating H_2O_2 (Halliwell et al. 2000). However, H_2O_2 is a relatively stable ROS that is capable of diffusing through cellular membranes. Thus, it is likely that endothelial cells are exposed to substantially more H_2O_2 generated from intimal SMC and inflammatory cells in the subendothelial space. While the concentration of H_2O_2 in atherosclerotic blood vessels is not known, levels of H_2O_2 can exceed 100 μmol/L in inflamed tissues (Halliwell et al. 2000). This may be pertinent to sites of intense inflammation in atherosclerotic blood vessels.

The mechanisms by which H_2O_2 induces vascular cell injury are not fully understood. H_2O_2 does not contain an unpaired electron and is therefore less reactive than many other ROS. Thus, mechanisms other than direct oxidant injury likely contribute to the cytotoxic effects of H_2O_2 in vascular cells. In this regard, there is increasing evidence that H_2O_2 can activate signaling pathways to stimulate ROS production in vascular cells. In SMC, H_2O_2 activates NADPH oxidase, resulting in the

production of $O_2^{\cdot-}$, and, consequently, oxidant injury (Li et al. 2001). However, it remains to be established whether this mechanism is also operative in endothelial cells. Moreover, in endothelial cells, H_2O_2 has been reported to stimulate NOS expression and activity (Cai et al. 2003b). It is plausible that H_2O_2-induced oxidative stress could lead to NOS uncoupling, which could in turn generate $O_2^{\cdot-}$ (Beckman et al. 1990; Pryor et al. 1995), although this has not been demonstrated experimentally. Finally, endothelial cells *in vivo* are exposed to shear stress, which modulates many key aspects of endothelial metabolism and function, including NO release (Griffith 2002), NOS expression (Dimmeler et al. 1999; Davis et al. 2004), copper/zinc superoxide dismutase expression (Dimmeler et al. 1999; Woodman et al. 1999), and the expression of other endothelial cell genes (Malek et al. 1995). However, whether shear stress modulates responses to H_2O_2 in endothelial cells is not known.

Accordingly, the current report investigates the mechanisms of H_2O_2-induced oxidative stress in porcine aortic endothelial cells. Experiments were performed under static and shear conditions in order to gain insight into the potential modulatory influence of shear on the actions of H_2O_2 in endothelial cells.

3.3 Methods

3.3.1 Porcine Aortic Endothelial Cell Culture

Porcine aortic endothelial cells (PAEC) were obtained from the University of Iowa Cardiovascular Research Center Cell Culture Facility. They were cultured in Medium 199 (Invitrogen, M199) supplemented with 1% Penicillin-Streptomycin (Invitrogen) and 10% Fetal Bovine Serum (Hyclone, FBS), with ascorbate levels in the physiologic range (Choy et al. 2003; Badrakhan et al. 2004). Cultures were maintained at 37°C with 95% humidity and 5% CO_2. Experiments were conducted in 24-well plates, at a density of 40,000 cells/well seeded 48-hours prior to experimentation. PAEC were utilized between passages 5-10.

3.3.2 Adenoviral Infection of PAEC

Adenoviral-mediated gene transfer was achieved by infection of endothelial cells (85-95% confluence) as previously described (Fang et al. 1998). Adenoviruses encoding human manganese superoxide dismutase (Ad5CMVSOD2, SOD2) and green fluorescent protein (Ad5CMVeGFP, eGFP) were acquired from the University of Iowa Gene Transfer Vector Core. Ad5CMVeGFP was used as a negative control for infection. PAEC were incubated with 125 PFU/cell SOD2/eGFP adenovirus in serum-free M199. To improve the efficiency of adenoviral uptake, the adenoviral stock was incubated at 37°C for 30 minutes prior to addition to cell cultures (Kossila et al. 2002). Non-infected PAEC were maintained under standard cell culture conditions as controls for all experimentation. After 3 hours, the virus was removed, the PAEC were washed with serum-free M199, and M199 supplemented with 1% Penicillin-Streptomycin and 2% FBS was added. Subsequent experimentation took place 48 hours after exposure to adenovirus.

3.3.3 Endothelial Cell Viability

PAEC were cultured as previously described. Forty-eight hours after infection, PAEC were incubated with 0-500 µmol/L H_2O_2 and/or 1 mmol/L 1,2-dihydroxybenzene-3,5-disulfonate (Sigma, Tiron) in serum-free M199 for 1.5 hours; control groups were incubated in serum-free M199. After incubation, 3 µmol/L propidium iodide (PI) was added to each sample and incubated for 8 minutes. Samples were then washed with serum-free and phenol-red free M199 and imaged.

3.3.4 Determination of Intracellular $O_2^{\cdot-}$

PAEC were exposed to vehicle or 60 µmol/L H_2O_2 in serum-free M199 for 1 hour. Incubation was continued for an additional 30 minutes in the presence of 2 µM dihydroethidium(Carter et al. 1994; Miller et al. 1998) (DHE, Molecular Probes). After incubation, PAEC were washed, placed in phenol-red-free and serum-free M199, and imaged. In previous studies, fluorescence intensity of the total culture was used as an

indicator of $O_2^{\cdot-}$ (Fang et al. 1998; Li et al. 2000; Li et al. 2001; Landmesser et al. 2002; Li et al. 2003). However, in this study, the presence or absence of fluorescence in individual cells was determined as a binary endpoint. The data were expressed as percentage of cells exhibiting fluorescent nuclei (Excitation: 530-560nm, Emission: 570-640nm). Also, 1 mmol/L Tiron was used as a positive control to verify that DHE fluorescence was produced by $O_2^{\cdot-}$.

3.3.5 Determination of Intracellular Nitric Oxide

To determine intracellular nitric oxide levels in PAEC in the presence or absence of exposure to H_2O_2, 4-amino-5-methylamino-2',7'- difluorofluorescein diacetate ((Itoh et al. 2000), DAF-FM diacetate, Molecular Probes) was used. PAEC were exposed to vehicle or 60 µmol/L H_2O_2 in serum-free M199 for 45 minutes. Incubation was continued for an additional 45 minutes in the presence of 4 µmol/L DAF-FM diacetate (Excitation: 495nm, Emission: 515nm). After incubation, PAEC were washed, placed in phenol-red-free and serum-free M199, and incubated an additional 15 minutes prior to fluorescent imaging. The presence or absence of fluorescence in individual cells was determined as a binary endpoint. The data were expressed as percentage of cells exhibiting fluorescent nuclei.

3.3.6 Superoxide Detection Assay under Shear

PAEC at a density of 500,000 cells/slide were cultured on Vitrogen (Cohesion, 0.032 mg/ml) coated glass microscope slides (Surgipath) as previously described (Sagnella et al. 2001). PAEC were exposed to a constant flow rate of 24.4 ml/min (8.2 dynes/cm^2) for 1.5 hours. Maintenance of PAEC at 37°C was achieved by use of a fluid reservoir contained in a constant temperature water bath (Precision). The flow system utilized an Immunetics flow chamber designed for one-dimensional shear (Li et al. 1996). PAEC were dynamically imaged during the course of the experiment using a Q-Imaging Retiga 1300 CCD camera to monitor cellular attachment and morphology with a Zeiss Axiovert S100 light microscope. PAEC were exposed to 100 µmol/L H_2O_2 in serum-free

and phenol-red-free M199; control PAEC were exposed to serum-free and phenol-red-free M199 without H_2O_2. An increase in the H_2O_2 concentration from that used in static experimentation was necessary due to peroxide scavenging by the polymer flow cell and tubing.

After 1 hour of incubation, 2 µmol/L dihydroethidium was added to the media reservoir. Images were collected at 0, 15, 30, 60, and 90 minutes and a fluorescent image was taken at 90 minutes (Excitation: 530-560nm, Emission: 570-640nm).

3.3.7 Additional Reagents and Materials

N^G-nitro-L-arginine methyl ester (L-NAME), a specific inhibitor of NOS, N^G-nitro-D-arginine methyl ester (D-NAME), a negative control for L-NAME, and apocynin, an inhibitor of NADPH oxidase, were obtained from Sigma. Menadione (Sigma), which generates $O_2^{\cdot-}$ by redox cycling through NADPH oxidase (Puntarulo et al. 1992), was utilized as a positive control for experiments with apocynin. L-sepiapterin (Cayman Chemical), a precursor of BH_4, was utilized to salvage BH_4 (Shen et al. 1988; Tarpey 2002; Blau et al. 2003).

3.3.8 Data Analysis

Image analysis was conducted with Slidebook 4 Imaging Software (Intelligent Imaging Innovations, Denver, CO). Statistical analysis was conducted using the Student's t-Test and the f-Test to determine the equality of variances. Values are reported as mean ± standard error of the mean (SEM).

3.4 Results

3.4.1 Porcine Aortic Endothelial Cells Produce O_2^- on Exposure to H_2O_2

In SMC, H_2O_2 and lipid hydroperoxide species such as 13-HPODE have been demonstrated to increase $O_2^{\cdot-}$ levels, which in turn contributes to H_2O_2-induced cytotoxicity (Li et al. 2000; Li et al. 2001; Li et al. 2003). To determine whether H_2O_2 increases $O_2^{\cdot-}$ levels in endothelial cells under static conditions, PAEC were treated with

vehicle or 60 µmol/L H_2O_2 for 1.5 hours, after which the presence of $O_2^{\cdot-}$ was examined using DHE. Following exposure of PAEC to H_2O_2, DHE fluorescence was detected in 53.6 ± 11.2% of nuclei. Following exposure to vehicle, only 4.9 ± 0.6% of nuclei exhibited DHE fluorescence (Figure 3.1 and Figure 3.2, n=12, $p<0.001$).

To confirm that the DHE fluorescence resulting from exposure to H_2O_2 was due to $O_2^{\cdot-}$, the human SOD2 gene was overexpressed using adenoviral-mediated gene transfer. In the absence of exposure to H_2O_2, overexpression of SOD2 had no significant effect on DHE fluorescence (Figure 3.1 and Figure 3.2). However, when SOD2-transfected PAEC were exposed to H_2O_2, only 5.7 ± 0.5% of nuclei exhibited fluorescence (n=12, $p<0.001$). In contrast, infection of PAEC with a control adenovirus expressing GFP did not result in a reduction in DHE fluorescence produced by H_2O_2.

To further confirm that the DHE fluorescence resulting from exposure to H_2O_2 was due to $O_2^{\cdot-}$, Tiron was used to scavenge $O_2^{\cdot-}$. In the absence of treatment with H_2O_2, exposure to Tiron had no significant effect on DHE fluorescence (Figure 3.1). However, when Tiron exposed PAEC were treated with H_2O_2, only 10.2 ± 4.5% of nuclei exhibited fluorescence (n=12, $p<0.001$). Together, these results confirmed that treatment with H_2O_2 increased intracellular $O_2^{\cdot-}$ levels in PAEC.

3.4.2 Contribution of $O_2^{\cdot-}$ to H_2O_2-induced Oxidative Stress in PAEC

To address the contribution of $O_2^{\cdot-}$ to H_2O_2-induced oxidant stress, propidium iodide staining was performed. Propidium iodide is actively transported out of living cells and accumulates in dead or dying cells (Massaro et al. 1989). In the absence of H_2O_2, 0.9 ± 0.2% of PAEC stained positively for propidium iodide (Figure 3.3). Exposure to 200 µmol/L H_2O_2 resulted in 4.4 ± 0.7% and 350 µmol/L H_2O_2 resulted in 5.1 ± 1.0% PAEC positive for propidium iodide, a significant increase over control ($p<0.01$, Figure 3.3). Exposure to 500 µmol/L H_2O_2 resulted in 27.6 ± 6.2% of PAEC staining positively for propidium iodide, and nearly five percent exhibited ruptured

cellular membranes, consistent with increased cytotoxicity (n=6, $p<0.001$; Figure 3.3). Treatment with Tiron markedly attenuated H_2O_2–induced cytotoxicity, as only 1.4 ± 0.4% of the PAEC exposed to Tiron stained positively for propidium iodide following exposure to 500 µmol/L H_2O_2.

To address the longer-term contribution of $O_2^{\cdot-}$ to H_2O_2-induced oxidant stress, propidium iodide staining was performed 24 hours after H_2O_2 exposure. In the absence of H_2O_2, 0.2 ± 0.1% of PAEC stained positively for propidium iodide (Figure 3.4). Exposure to 60 µmol/L H_2O_2 resulted in 84.5 ± 10.1% PAEC positive for propidium iodide, a significant increase over control ($p<0.001$, Figure 3.4). PAEC positive for propidium iodide was reduced to 6.9 ± 3.3% with exposure to Tiron and H_2O_2. These findings suggest that $O_2^{\cdot-}$ plays a significant role in H_2O_2-induced cytotoxicity of PAEC under static conditions.

3.4.3 NOS is an Enzymatic Source of $O_2^{\cdot-}$ in H_2O_2-treated PAEC

H_2O_2 has been shown to activate NOS, and NOS can generate $O_2^{\cdot-}$ when essential cofactors such as tetrahydrobiopterin (BH_4) become oxidized. This prevents the enzyme from transferring electrons to its physiological substrate L-arginine (termed "uncoupling" of NOS) (Drummond et al. 2000; Thomas et al. 2002; Cai et al. 2003b; Kuzkaya et al. 2003; Landmesser et al. 2003). We therefore investigated the role of NOS in H_2O_2-induced increase in $O_2^{\cdot-}$ levels in PAEC. First, we examined NO levels in PAEC using a fluorescent probe, DAF-FM diacetate. In the absence of treatment with H_2O_2, 2.1 ± 0.6% of PAEC exhibited DAF-FM fluorescence. Treatment with 60 µM H_2O_2 for 1.5 hours produced a marked increase in DAF-FM fluorescence, with 59.1 ± 4.3% of PAEC exhibiting DAF-DM fluorescence (n=12, $p<0.001$ compared with no H_2O_2 treatment). This was reduced to 24.9 ± 8.1% in the presence of N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS activity (n=6, $p<0.001$, (Pritchard et al. 1995; Munzel et al.

2000; Pritchard et al. 2001)). These findings suggest that treatment with H_2O_2 resulted in increased NOS activity in PAEC.

Further, when L-NAME treated PAEC were exposed to H_2O_2, there was a marked diminution of nuclear DHE fluorescence, indicative of reduced intracellular $O_2^{\cdot-}$ (Figure 3.5). With L-NAME treatment, only 19.3 ± 3.2% of H_2O_2-exposed PAEC exhibited fluorescent nuclei as compared to with 52.1 ± 5.7% of vehicle-treated, H_2O_2-exposed PAEC (n=9, $p<0.001$). In contrast, treatment with D-NAME had no effect on H_2O_2-induced $O_2^{\cdot-}$ in PAEC (data not shown). These findings suggest that NOS plays an important role in H_2O_2-induced increases in $O_2^{\cdot-}$ in PAEC (Massaro et al. 1989).

Next, when L-sepiapterin (15 μmol/L) treated PAEC were exposed to H_2O_2, there was a distinct reduction of nuclear DHE fluorescence, indicative of reduced intracellular $O_2^{\cdot-}$ (Figure 3.6). With L-sepiapterin treatment, only 36.0 ± 7.2% of H_2O_2-exposed PAEC exhibited fluorescent nuclei as compared to with 68.5 ± 5.2% of vehicle-treated, H_2O_2-exposed PAEC (n=9, $p<0.003$). These findings suggest that NOS plays an important role in H_2O_2-induced increases in $O_2^{\cdot-}$ in PAEC (Massaro et al. 1989) and that L-sepiapterin rescues uncoupled NOS.

3.4.4 NADPH Oxidase is an Enzymatic Source of $O_2^{\cdot-}$ in H_2O_2-treated PAEC

Besides NOS, endothelial cells contain other enzymes, such as NADPH oxidase, that are capable of generating $O_2^{\cdot-}$. Moreover, levels of H_2O_2 similar to those used in the present study were shown to activate NADPH oxidase in SMC, leading to $O_2^{\cdot-}$ production and enhanced cytotoxicity (Li et al. 2001). We therefore investigated the role of NADPH oxidase in H_2O_2-induced increases in $O_2^{\cdot-}$ in PAEC, using apocynin as a pharmacological inhibitor of NADPH oxidase. First, we verified the efficacy of apocynin to inhibit NADPH oxidase in PAEC by exposing the cells to menadione, an activator of NADPH oxidase (Afanas'ev et al. 1990; Puntarulo et al. 1992; Suzuki et al. 1999). Treatment of PAEC with 250 μmol/L apocynin did not affect basal DHE fluorescence (in the absence

of exposure to menadione). Exposure to menadione resulted in marked increases in DHE fluorescence, 75% of which was blocked by apocynin, confirming that the inhibitor effectively blocked NADPH oxidase activity (data not shown).

Next, we tested whether treatment with apocynin could block the H_2O_2-induced increase in $O_2^{\cdot-}$ in PAEC. As previously described, treatment with H_2O_2 produced a marked increase in DHE fluorescence in PAEC, indicative of increased $O_2^{\cdot-}$ (51.5 ± 3.7% of H_2O_2-exposed PAEC exhibited DHE fluorescence). When apocynin-treated PAEC were exposed to H_2O_2, there was a significant inhibition of nuclear DHE fluorescence, indicative of reduced intracellular $O_2^{\cdot-}$ (Figure 3.5). With apocynin treatment, only 23.2 ± 6.1% of H_2O_2-exposed PAEC exhibited DHE fluorescence (n=9, $p<0.01$ as compared with no apocynin treatment). These findings suggest that in addition to NOS, NADPH oxidase contributes to the H_2O_2-induced increase in $O_2^{\cdot-}$ in PAEC.

3.4.5 The Effects of H_2O_2 on $O_2^{\cdot-}$ in PAEC under Physiologic Shear

Endothelial cells *in vivo* are exposed to shear stress, which modulates endothelial ROS production and bioactivity. Therefore, the effects of H_2O_2 on PAEC were probed under shearing conditions. PAEC on Vitrogen coated glass slides were subjected to oscillatory laminar flow at 24.4 ml/min (8.2 dynes/cm^2). The circulating medium (medium M199 without phenol red) contained 2 µmol/L dihydroethidium to detect intracellular $O_2^{\cdot-}$. Figures 3.7 & 3.8 show the results of these experiments. Exposure to 100 µmol/L H_2O_2 for 1.5 h resulted in 50.3 ± 4.1% of PAEC exhibiting DHE fluorescence as compared to 1.6 ± 0.9% in vehicle-treated PAEC (n=4, $p<0.01$), suggesting increased intracellular $O_2^{\cdot-}$.

Overexpression of the human SOD2 gene was conducted to confirm that treatment with H_2O_2 increased intracellular $O_2^{\cdot-}$ in PAEC under shear conditions. When SOD2-transfected PAEC were exposed to H_2O_2, 26.4 ± 3.2% of nuclei exhibited fluorescence under shear (n=4, $p<0.02$). Moreover, when Tiron exposed PAEC were

exposed to H_2O_2, only 16.2 ± 7.6% of nuclei exhibited fluorescence (n=5, $p<0.01$). PAEC attachment is reduced with exposure to H_2O_2 and this effect is abated with SOD2 overexpression. These results confirm that treatment with H_2O_2 under oscillatory laminar shear resulted in increased intracellular $O_2^{\cdot-}$ in PAEC, suggesting that our observations with H_2O_2 could be relevant to endothelial $O_2^{\cdot-}$ production *in vivo*.

3.5 Discussion

The current study probes the effects of H_2O_2 on PAEC under static and shearing conditions. Three major conclusions can be drawn from this study: 1) In PAEC, H_2O_2 exposure increased intracellular $O_2^{\cdot-}$, which caused cytotoxicity to the endothelial cells; 2) This mechanism was conserved under physiologic shear. 3) Both NOS and NADPH oxidase contributed to the increased $O_2^{\cdot-}$ induced by H_2O_2 in PAEC.

The stimuli for ROS production in the vasculature are diverse and include cytokines and growth factors such as angiotensin II and thrombin; metabolic factors such as glucose, fatty acids, and homocysteine; immunostimulatory molecules such as endotoxin; and mechanical forces (Cai et al. 2002; Cai et al. 2003a; Hwang et al. 2003; McNally et al. 2003; Taniyama et al. 2003). Reactive oxygen species include a number of structurally distinct molecules with unique chemical reactivity and biological effects. H_2O_2, a relatively stable ROS, has attracted considerable interest as a modulator of vascular cell function and viability in physiologic and pathologic states. Endogenous H_2O_2 has been shown to be an important signaling molecule leading to increased DNA replication and proliferation in smooth muscle cells and endothelial cells (Brown et al. 1999). Also, endogenous H_2O_2 has been demonstrated to regulate vascular smooth muscle cell tone in several species and experimental models (Rabelo et al. 2003; Yada et al. 2003).

In contrast to the low levels of endogenous H_2O_2 that modulate physiologic cellular functions, high levels of H_2O_2, such as is observed in inflammatory states, generally cause cellular dysfunction and/or cytotoxicity. Interestingly, under these

conditions, H_2O_2-induced oxidant injury may be mediated indirectly through modulation of metabolic pathways and intracellular signaling cascades. For example, endothelial cell apoptosis induced by H_2O_2 was reported to require increased cellular iron uptake via a transferrin receptor dependent mechanism (Tampo et al. 2003). Further, H_2O_2-induced apoptosis in smooth muscle cells was shown to depend upon activation of protein kinase C (PKC) (Li et al. 1999). In bovine aortic endothelial cells, inhibition of PKC delta blocked the accumulation of p53 and subsequent apoptosis induced by H_2O_2 (Niwa et al. 2002). In rat aortic smooth muscle cells, H_2O_2-induced apoptosis was found to be dependent upon activation of NADPH oxidase and production of $O_2^{\cdot -}$ (Li et al. 2001).

In this study, we investigated the mechanisms whereby exogenously applied H_2O_2 promotes oxidative stress and cytotoxicity in endothelial cells. As was observed in rat aortic SMC (Li et al. 2001), we found that in PAEC, application of H_2O_2 increased $O_2^{\cdot -}$, which mediated oxidant injury. However, while NADPH oxidase was the enzymatic source of H_2O_2-induced $O_2^{\cdot -}$ in SMC, the results of this study suggest that both NOS and NADPH oxidase contribute to H_2O_2-induced $O_2^{\cdot -}$ in PAEC. The observation that NOS contributes to H_2O_2-induced $O_2^{\cdot -}$ in PAEC is novel and somewhat unexpected. While H_2O_2 has been shown to activate NOS in endothelial cells (Cai et al. 2003b), it has not been demonstrated to uncouple the enzyme to generate $O_2^{\cdot -}$. The mechanisms responsible for NOS uncoupling are not fully understood but likely involve oxidation of essential enzyme cofactors such as tetrahydrobiopterin (BH_4) (Vasquez-Vivar et al. 2003). Addition of L-sepiapterin in culture has been shown to salvage BH_4 (Nichol et al. 1983), allowing for NOS production of NO rather then $O_2^{\cdot -}$ in the presence of $ONOO^-$.

In cell-free systems, peroxynitrite, but not H_2O_2, is capable of oxidizing BH_4 (Milstien et al. 1999). Application of peroxynitrite to cultured endothelial cells also produced NOS uncoupling by oxidizing BH_4 (Kuzkaya et al. 2003). Moreover, this mechanism may contribute to vascular $O_2^{\cdot -}$ production *in vivo* in some pathological states (Landmesser et al. 2003). Although we did not measure peroxynitrite levels in the

present study, our data suggest that exposure of PAEC to H_2O_2 resulted in simultaneous increases in NO and $O_2^{\cdot-}$, which have been demonstrated to rapidly react to form peroxynitrite. The reduction in $O_2^{\cdot-}$ by L-sepiapterin suggests that $ONOO^-$ is present and uncoupling NOS with exposure to H_2O_2. This may explain why a relatively weak oxidant like H_2O_2 is presumably capable of oxidizing BH_4 to uncouple NOS. However, further experimentation is required to make this conclusion definitively.

While our results suggest that application of high concentrations of H_2O_2 to endothelial cells caused sufficient oxidant stress to produce NOS uncoupling, Shimizu et al. reported concentrations of H_2O_2 similar to those used in the present study increased levels of BH_4 and induced NO production by NOS (Shimizu et al. 2003). However, the latter study was performed in mouse brain microvascular endothelial cells (MBMEC). Moreover, the increases in biopterin levels were observed only when MBMEC were exposed to H_2O_2 for eight hours or more. Our study utilized a shorter time course (1.5 hours) compared with 8 hours, and our endothelial cells were derived from a different species and vascular bed. These and other differences in experimental conditions likely account for the divergent results obtained in the two studies.

Endothelial cells *in vivo* are continuously exposed to shear stress, which has an important impact on cellular structure, function, and metabolism. Accordingly, we also examined responses to H_2O_2 in PAEC exposed to physiologic shear. As was observed under static culture conditions, exposure of PAEC to H_2O_2 resulted in increased intracellular $O_2^{\cdot-}$ that was reduced by both Tiron treatment and MnSOD gene transfer. Importantly, overexpression of MnSOD protected PAEC from H_2O_2-induced oxidative injury when the cells were exposed to physiologic shear improving cellular attachment. These results indicate that H_2O_2 could contribute to endothelial cell oxidative stress *in vivo* by increasing $O_2^{\cdot-}$. Such a mechanism could help to explain the beneficial effects of superoxide dismutase mimics on vascular oxidative stress observed in numerous experimental studies.

3.6 Acknowledgements

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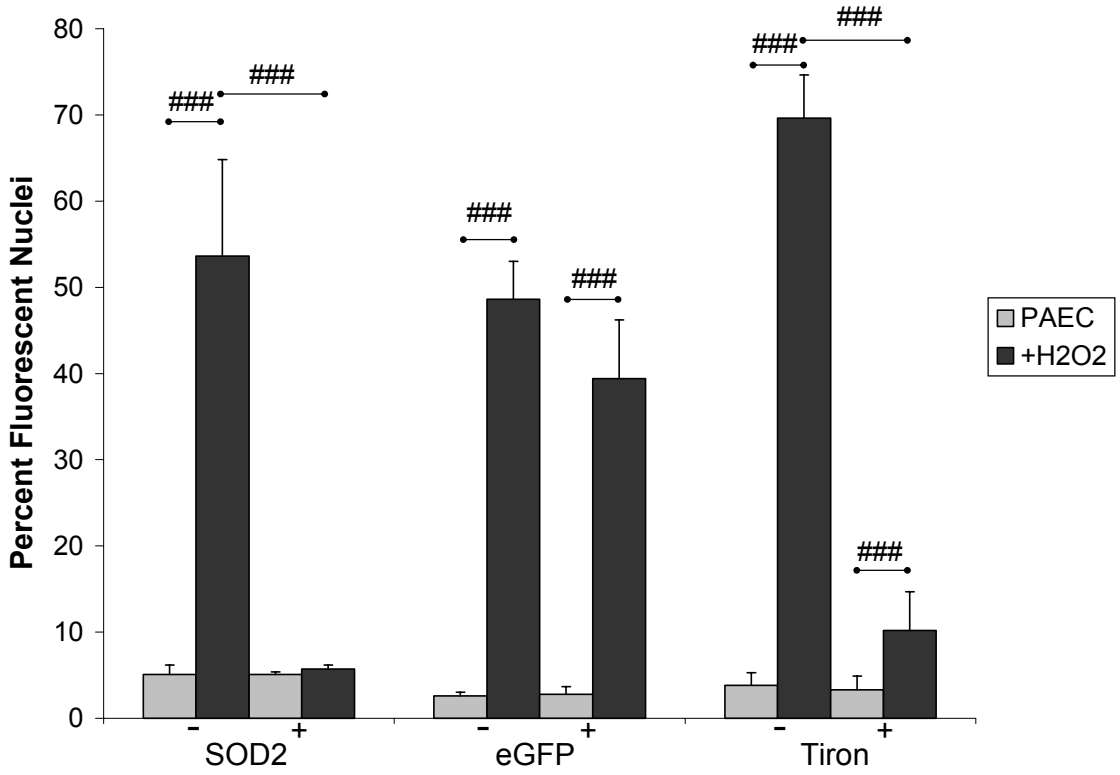

Figure 3.1: H_2O_2 Increases $O_2^{\cdot-}$ in PAEC. PAEC under static conditions were exposed to 0 (control) or 60 µmol/L H_2O_2 for 1.5 h, after which intracellular $O_2^{\cdot-}$ was examined using fluorescent microscopy with DHE. Prior to experimentation, some cells were infected with adenoviral vectors expressing GFP (as a control) or MnSOD, while other cells were treated with Tiron, which scavenges $O_2^{\cdot-}$. Data are expressed as mean ± SEM (###$p<0.001$).

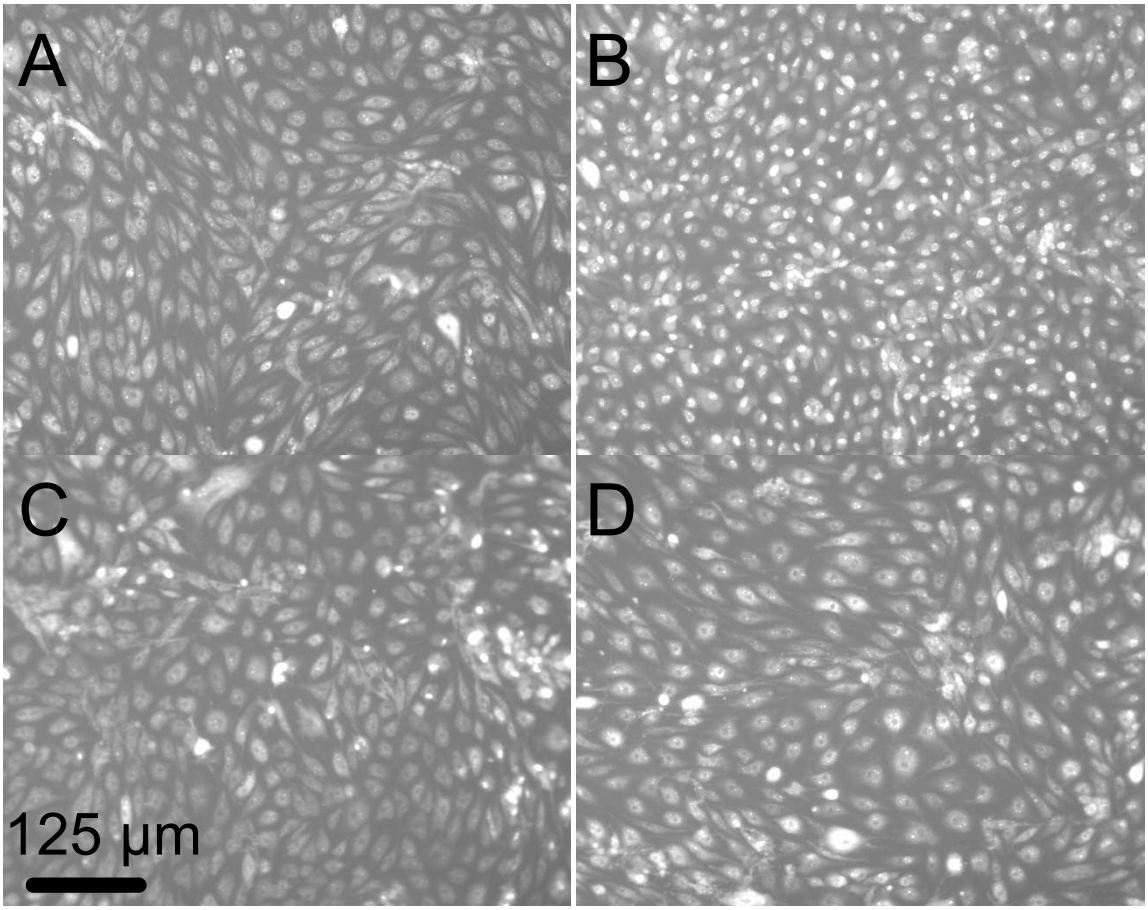

Figure 3.2: H_2O_2 Increases $O_2^{\cdot-}$ in PAEC. Representative fluorescent micrograph showing the effects of H_2O_2 on intracellular $O_2^{\cdot-}$ in PAEC under static conditions (10X magnification). Cells were exposed to 0 (A & C) or 60 µmol/L H_2O_2 (B & D), in the absence (A & B) or presence (C & D) of MnSOD overexpression. After 1.5 h, cells were examined by fluorescent microscopy, as described above. Results are representative of those obtained in 12 experiments.

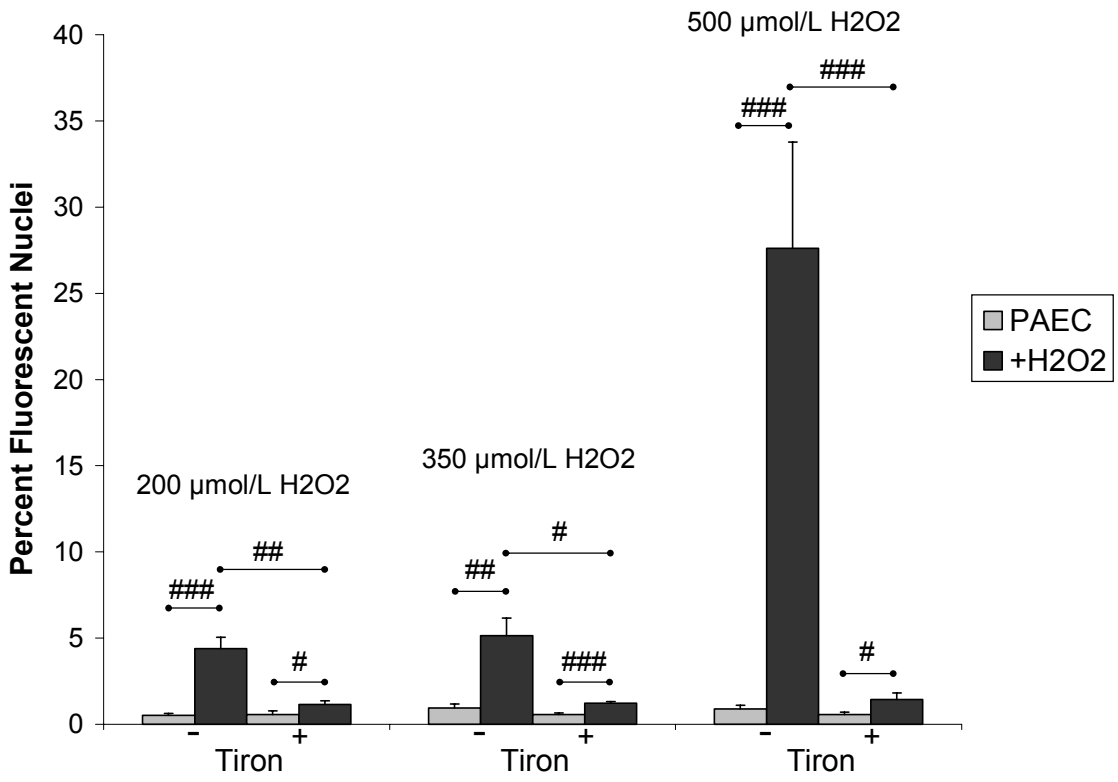

Figure 3.3: $O_2^{\cdot-}$ Contributes to H_2O_2-induced Cytotoxicity in PAEC. PAEC under static conditions were exposed to 0 (control), 200 µmol/L H_2O_2, 350 µmol/L H_2O_2, or 500 µmol/L H_2O_2 for 1.5 h in the absence or presence of Tiron, after which viability was examined using fluorescent microscopy with propidium iodide. Data are expressed as mean ± SEM with (###p<0.001, ##p<0.01, or #p< 0.05).

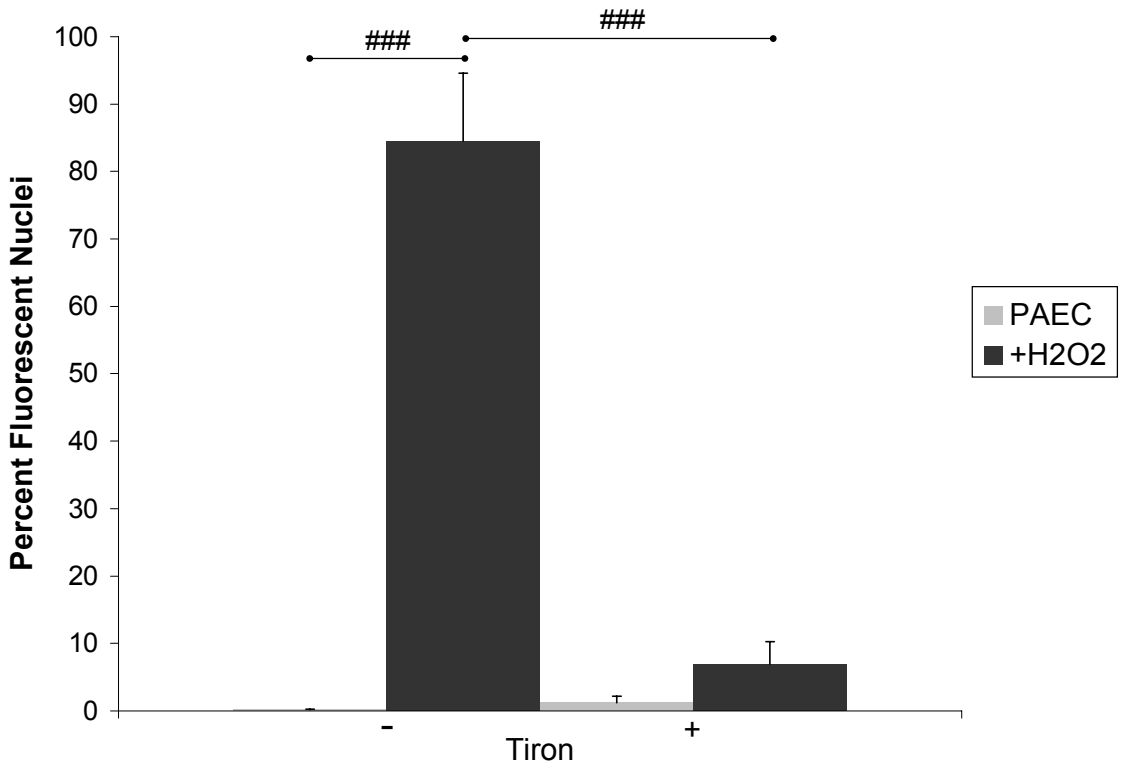

Figure 3.4: $O_2^{\cdot-}$ Contributes to H_2O_2-induced Cytotoxicity in PAEC after 24 Hour Incubation. PAEC under static conditions were exposed to 0 (control), 60 µmol/L H_2O_2 for 1.5 h in the absence or presence of Tiron. After 22.5 h incubation, viability was examined using fluorescent microscopy with propidium iodide. Data are expressed as mean ± SEM with (###$p<0.001$).

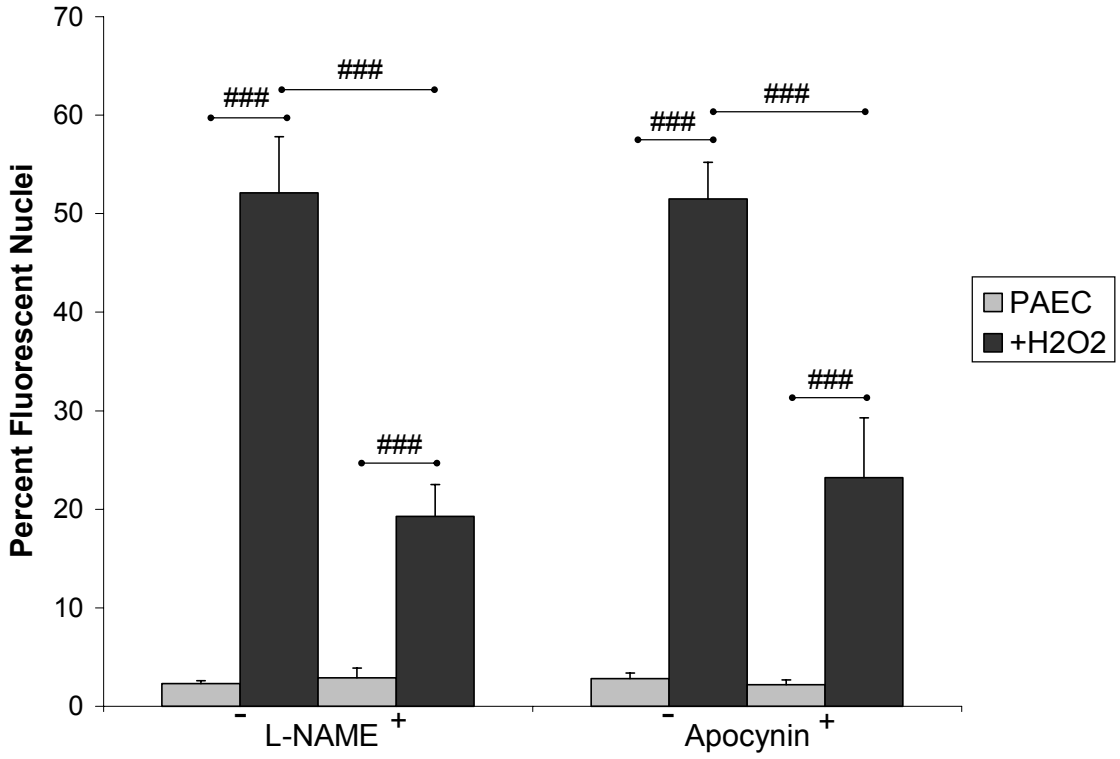

Figure 3.5: L-NAME and Apocynin Attenuate the Increase in $O_2^{\cdot-}$ Induced by H_2O_2 in PAEC. PAEC under static conditions were exposed to 0 (control) or 60 µmol/L H_2O_2 and either 1 mmol/L L-NAME (NOS inhibitor) or 250 µmol/L apocynin (NADPH oxidase inhibitor) for 1.5 h, after which intracellular $O_2^{\cdot-}$ was examined using fluorescent microscopy with DHE. Data are expressed as mean ± SEM with (###p<0.001).

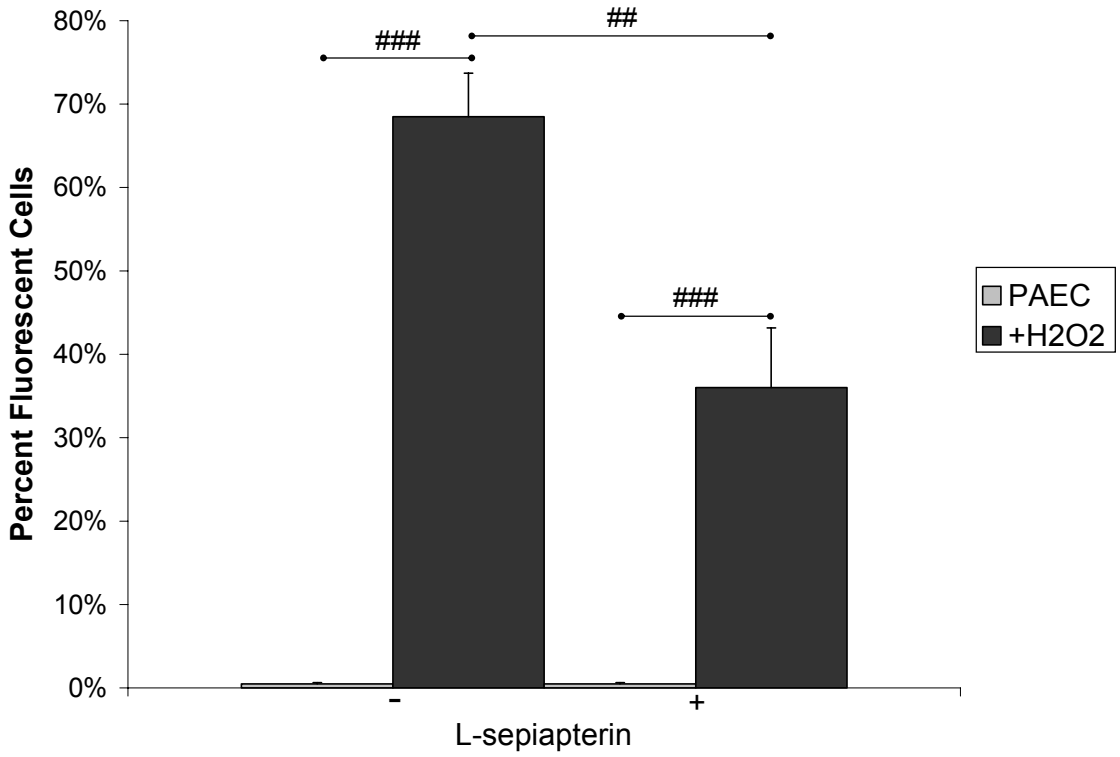

Figure 3.6: L-Sepiapterin Attenuates the Increase in $O_2^{\cdot-}$ Induced by H_2O_2 in PAEC. PAEC under static conditions were exposed to 0 (control) or 60 µmol/L H_2O_2 for 1.5 h with or without 15 µmol/L L-sepiapterin, after which intracellular $O_2^{\cdot-}$ was examined using fluorescent microscopy with DHE. Data are expressed as mean ± SEM with (###$p<0.001$ or ##$p< 0.01$).

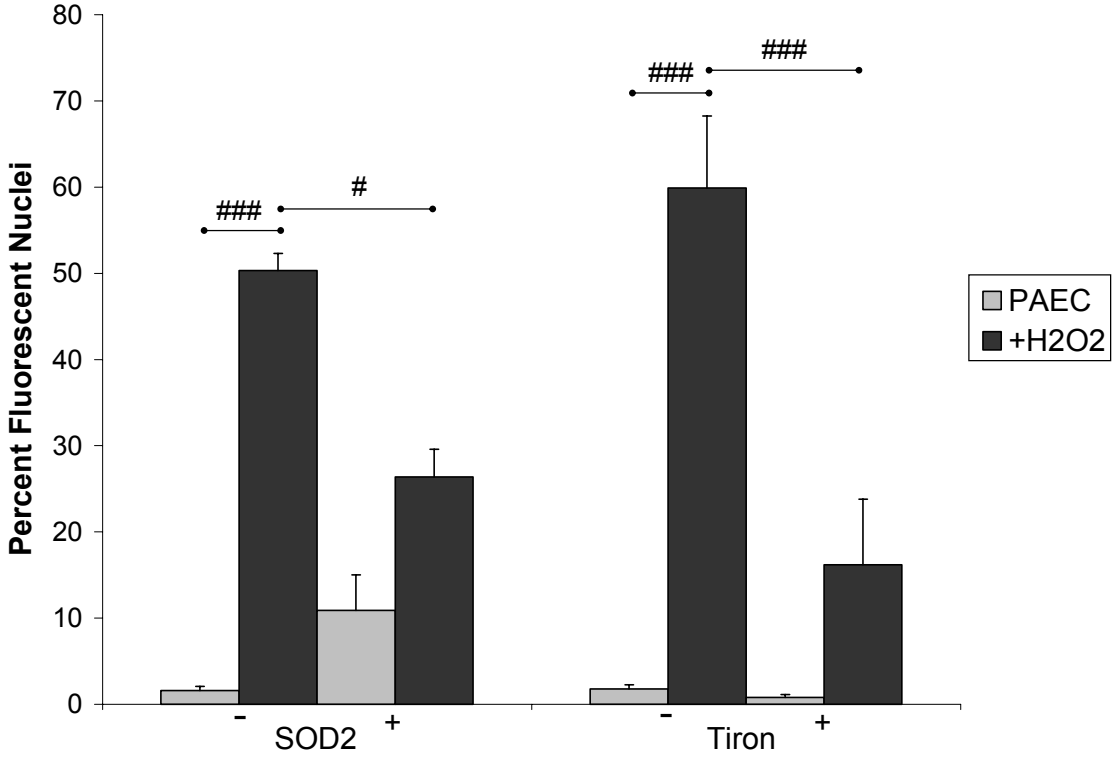

Figure 3.7: Effects of H_2O_2 on PAEC under Shearing Conditions. PAEC under shear conditions (8.2 dynes/cm^2) were exposed to 0 (control) or 100 µmol/L H_2O_2 for 1.5 h, after which intracellular $O_2^{\cdot-}$ was examined using fluorescent microscopy with DHE. Prior to experimentation, some cells were infected with adenoviral vectors expressing MnSOD, while other cells were treated with Tiron, which scavenges $O_2^{\cdot-}$. Data are expressed as mean ± SEM with (###$p<0.001$ or #$p< 0.05$).

Figure 3.8: Time Course of the Effects of H_2O_2 on PAEC under Shearing Conditions. Representative time course of light micrographs (0, 30, 60, 90 minutes) and a fluorescent micrograph (90 minutes) showing the effects of H_2O_2 on intracellular $O_2^{\cdot-}$ in PAEC under shearing conditions (10X magnification). Cells were exposed to 0 (A & C) or 100 µmol/L H_2O_2 (B & D), in the absence (A & B) or presence (C & D) of MnSOD overexpression. After 1.5 h, cells were examined by fluorescent microscopy, as described previously.

CHAPTER 4
MECHANISMS OF H_2O_2-INDUCED OXIDATIVE STRESS IN ENDOTHELIAL CELLS EXPOSED TO PHYSIOLOGIC SHEAR STRESS

4.1 Abstract

Hydrogen peroxide is produced by vascular and inflammatory cells for cell signaling and induces oxidative stress. H_2O_2 may also contribute to endothelial cell dysfunction and to the formation and progression of vascular disease. In smooth muscle cells, H_2O_2 induces production of $O_2^{\cdot-}$ via NADPH oxidase. However, the mechanisms whereby H_2O_2 induces oxidative stress in endothelial cells are not well understood, though $O_2^{\cdot-}$ may play a role. A recent study documented increased $O_2^{\cdot-}$ in endothelial cells exposed to H_2O_2 via uncoupled NOS and NADPH oxidase. To assess responses to H_2O_2 in PAEC under shearing conditions, a constant flow rate of 24.4 ml/min was applied to produce physiologically relevant shear stress (8.2 dynes/cm^2). Treatment with 100 μmol/L H_2O_2 has been shown to increase intracellular $O_2^{\cdot-}$ in PAEC. Here we demonstrate that L-NAME, an inhibitor of nitric oxide synthase (NOS), and apocynin, an inhibitor of NADPH oxidase, reduce $O_2^{\cdot-}$ in PAEC treated with H_2O_2 under physiologic shear. This suggests that both NOS and NADPH oxidase contribute to H_2O_2-induced $O_2^{\cdot-}$ in PAEC. Co-inhibition of NOS and NADPH oxidase also reduced intracellular $O_2^{\cdot-}$ under both static and shear culture. We conclude that H_2O_2-induced oxidative stress in endothelial cells exhibits increased intracellular $O_2^{\cdot-}$ through NOS and NADPH oxidase under shear. Co-inhibition of NOS and NADPH with exposure to H_2O_2 is additive under shear culture, yet non-additive under static culture, compared to the enzymes individual contributions. These findings suggest a complex interaction between H_2O_2 and oxidant-generating enzymes that may contribute to endothelial dysfunction in cardiovascular diseases.

4.2 Introduction

Hydrogen peroxide (H_2O_2) is a ROS that has received recent attention for its possible role in the etiology of a number of cardiovascular diseases, including hypertension and atherosclerosis. H_2O_2 is a byproduct of mitochondrial processes (Poderoso et al. 1998a) and superoxide dismutase (Griendling et al. 2000a; Salvemini et al. 2002). In previous studies, we found that higher than physiologic concentrations of H_2O_2 result in higher intracellular superoxide ($O_2^{\cdot-}$) in endothelial cells (Coyle et al. 2004). A number of processes may be responsible for heightened systemic H_2O_2 concentrations, including increased cellular production, concentration of H_2O_2 in flow disturbances, and activation and recruitment of inflammatory cells.

Oxidative stress, triggered partially by reactive oxygen species (ROS) causes endothelial cell dysfunction, cellular injury, and tissue damage, which can contribute to the pathology of atherosclerosis (Kuzkaya et al. 2003) and other vascular diseases (Cai et al. 2000; Landmesser et al. 2001; Uemura et al. 2001; Cooke 2004). $O_2^{\cdot-}$ and other ROS may have a key role in the early development of atherosclerosis, even prior to lesion development as increases in ROS are associated with many disease risk factors (Kuzkaya et al. 2003).

Recent studies have documented various effects of H_2O_2 in the vascular milieu (Griendling et al. 2000a; Li et al. 2001; Landmesser et al. 2002; Cai et al. 2003b), including smooth muscle cell and endothelial cell migration, and activation of inflammatory signaling mechanisms such as NF-κB (Wolin et al. 2002; Cai et al. 2003b; Landmesser et al. 2003; Davis et al. 2004). H_2O_2 has also been found to stimulate increased eNOS expression, eNOS activity, and nitric oxide production in endothelial cells (Drummond et al. 2000; Thomas et al. 2002; Cai et al. 2003b). Its ability to induce both nitric oxide (NO) and $O_2^{\cdot-}$ production makes H_2O_2 a potent oxidative stimulus as it provides the components necessary for peroxynitrite formation (NO and $O_2^{\cdot-}$ interaction, (Beckman et al. 1990; Pryor et al. 1995). This suggests a dual role for NOS if it should

become uncoupled, allowing for both $O_2^{\cdot-}$ and NO production via NOS (Milstien et al. 1999; Vasquez-Vivar et al. 2002; Kuzkaya et al. 2003; Landmesser et al. 2003; Vasquez-Vivar et al. 2003).

H_2O_2 has also been shown to contribute to vascular cell cytotoxicity in both smooth muscle and endothelial cells. Increased $O_2^{\cdot-}$ and smooth muscle cell toxicity have been documented with SMC exposure to H_2O_2 via an NADPH oxidase dependant mechanism (Li et al. 2001). The mechanisms through which H_2O_2 induces endothelial cytotoxicity are not fully understood. However, increased intracellular $O_2^{\cdot-}$ may play a role (Kapiotis et al. 1997). Recent studies in our laboratory have documented increased intracellular $O_2^{\cdot-}$ in H_2O_2-exposed endothelial cells as well as increased cytotoxicity under both shear and static conditions. NOS and NADPH oxidase were identified as important contributors to increased $O_2^{\cdot-}$ in PAEC exposed to H_2O_2 under static culture (Coyle et al. 2004).

As multiple endothelial cell mechanisms including nitric oxide release (Griffith 2002), nitric oxide synthase expression (Dimmeler et al. 1999; Davis et al. 2004), copper/zinc superoxide dismutase expression (Dimmeler et al. 1999; Woodman et al. 1999), and endothelial cell gene expression (Malek et al. 1995) are shear dependant. Experimentation under shear stress is necessary in understanding the disease associated pathways in endothelial cells.

While we previously probed the source of H_2O_2-induced superoxide through both the NOS and NADPH mechanisms, the interrelationship between the two pathways was not probed. This study describes the effects of H_2O_2 on both pathways simultaneously, under static conditions and physiologic shear.

4.3 Materials and Methods

4.3.1 Porcine Aortic Endothelial Cell Culture

Porcine aortic endothelial cells (University of Iowa Cardiovascular Research Center, PAEC) were used from passage 6-8. Sub-populations of PAEC were maintained

in Medium 199 (Invitrogen, M199) supplemented with 10% Fetal Bovine Serum (Invitrogen, FBS) and 1% Penicillin-Streptomycin (Invitrogen). Forty-eight hours prior to experimentation, PAEC were plated on 24-well plates at a density of 40,000 cells/ml for static experimentation or Vitrogen (Cohesion, 0.032 mg/ml, (Sagnella et al. 2001)) coated glass microscopy slides (Surgipath) at a density of 250,000 cells/ml for shear experiments.

4.3.2 Adenoviral Infection of PAEC

Adenoviral-mediated gene transfer was utilized as previously described (Fang et al. 1998; Coyle et al. 2004). Briefly, PAEC (85-95% confluency) were incubated with 125 PFU/cell of Ad5CMVeNOS (NOS3) or Ad5CMVeGFP (eGFP) for 3 hours in M199. Controls were incubated in M199 during infection. The viral solution was removed and PAEC were washed with M199 and incubated for an additional 45 hours in M199 supplemented with 2% FBS and 0.2% Penicillin-Streptomycin. Adenoviral loading efficiency was improved with incubation of the adenoviral stock for 30 minutes at 37°C prior to cell exposure (Kossila et al. 2002). eNOS encodes for human endothelial nitric oxidase synthase and eGFP encodes for green fluorescent protein.

4.3.3 Determination of Intracellular $O_2^{\cdot-}$

PAEC were incubated for 30 minutes with 2 µmol/L dihydroethidium ((Carter et al. 1994; Miller et al. 1998) DHE, Invitrogen) after 1 hour incubation with 60 µmol/L H_2O_2 in M199 or M199 as a control. After incubation with DHE, PAEC were washed in phenol-red-free M199 and imaged with a Zeiss fluorescent microscopy utilizing a rhodamine filter cube. As previously described (Coyle et al. 2004), cellular fluorescence was determined as a binary endpoint with an intensity threshold.

4.3.4 Intracellular $O_2^{\cdot-}$ Detection under Shear

PAEC were exposed to a flow rate of 24.4 ml/min (8.2 dynes/cm^2) for 1.5 hours. PAEC were maintained with a fluid reservoir in a water bath maintained at 37°C (Precision). An Immunetics flow chamber designed for one-dimensional shear (Li et al.

1996) was utilized in the flow system along with a cartridge pump and fluid reservoir (Figure 4.1). PAEC were dynamically imaged throughout the experiment with a Q-Imaging Retiga 1300 CCD camera and a Zeiss Axiovert S100 light microscope to monitor cellular morphology. PAEC were exposed to 100 µmol/L H_2O_2 in phenol red free M199, while control PAEC were exposed to phenol red free M199. An increase in H_2O_2 concentration was necessary due to scavenging of H_2O_2 by the shear system components.

After 1 hour of incubation, 2 µmol/L dihydroethidium was added to the media reservoir. Images were collected automatically every 2.5 minutes for 90 minutes followed by a fluorescent image at 90 minutes using a rhodamine cube.

4.3.5 Additional Reagents and Materials

N^G-nitro-L-arginine methyl ester (L-NAME), a specific inhibitor of NOS at the haem site (Pritchard et al. 1995; Munzel et al. 2000; Pritchard et al. 2001; Stepp et al. 2002) and apocynin, an inhibitor of NADPH oxidase protein complex formation (Jaimes et al. 2004), were obtained from Sigma and utilized in inhibition studies.

4.3.6 Image Analysis

Analysis of fluorescent images was carried out with Slidebook 4 Imaging Software (Intelligent Imaging Innovations, Denver, CO). Statistical analyses were conducted with the Student's t-Test and the f-Test to determine equality of variances. Experimental values are reported as mean ± standard error of the mean (SEM).

4.4 Results

4.4.1 NOS and NADPH Oxidase Co-inhibition

The combined effects of NOS and NADPH oxidase in H_2O_2-induced increases in intracellular $O_2^{\cdot-}$ in PAEC were investigated using L-NAME, which blocks both NO and $O_2^{\cdot-}$ production by NOS (Pritchard et al. 1995; Munzel et al. 2000; Pritchard et al. 2001) and apocynin, a pharmacological inhibitor of NADPH oxidase (Jaimes et al. 2004). Combination of L-NAME and apocynin slightly increased basal DHE fluorescence in the

absence of H_2O_2 to 6.2 ± 1.5% from 2.1 ± 0.9% (n=12, $p<0.05$). However, when L-NAME and apocynin treated PAEC were exposed to H_2O_2, 60.8 ± 3.3% of H_2O_2-exposed PAEC exhibited fluorescent nuclei as compared to 93.8 ± 1.7% of vehicle-treated, H_2O_2-exposed PAEC (n=12, $p<0.001$). A discernible attenuation of nuclear DHE fluorescence was observed with the inhibitors and H_2O_2 exposure compared to H_2O_2 exposed PAEC, indicative of reduced intracellular $O_2^{\cdot-}$ (Figure 4.2). These findings suggest that NOS and NADPH oxidase inhibition of $O_2^{\cdot-}$ in PAEC is non-additive compared to their individual contributions under static conditions (Coyle et al. 2004).

4.4.2 eNOS Gene Transfer Does Not Affect $O_2^{\cdot-}$

To probe the role of eNOS in H_2O_2-induced increases in intracellular $O_2^{\cdot-}$ in PAEC, the human eNOS gene was overexpressed. Adenoviral-mediated infection with eNOS resulted in a 3-fold increase in NO compared to control as determined by the Greiss assay (data not shown). When eNOS-transfected PAEC were exposed to H_2O_2, 57.7 ± 6.0% of nuclei exhibited fluorescence compared with 47.9 ± 5.0% of vehicle-treated, H_2O_2-exposed PAEC (Figure 4.3; n=24, $p>0.25$). Thus, eNOS adenoviral-mediated gene transfer does not significantly affect $O_2^{\cdot-}$ in PAEC exposed to H_2O_2 under static conditions.

The role of eNOS in NOS contribution to increased H_2O_2-induced increases in $O_2^{\cdot-}$ in PAEC was also investigated under shear conditions. When infected PAEC (eNOS) were exposed to H_2O_2, 30.8 ± 4.3% of nuclei exhibited nuclear fluorescence compared to 34.6 ± 7.9% of vehicle-treated, H_2O_2-exposed PAEC (Figure 4.3; n=7, $p>0.25$). These data suggests that upregulation of eNOS does not appreciably affect $O_2^{\cdot-}$ in PAEC exposed to H_2O_2 under both static and shear culture conditions.

4.4.3 NOS is an Enzymatic Source of $O_2^{\cdot-}$ in H_2O_2-treated PAEC Under Physiologic Shear

H_2O_2 has been shown to activate NOS, and NOS can generate $O_2^{\cdot-}$ when tetrahydrobiopterin becomes oxidized via peroxynitrite ($ONOO^-$, (Cai et al. 2003b;

Kuzkaya et al. 2003; Landmesser et al. 2003). Oxidation prevents NOS from transferring electrons to its physiologic substrate L-arginine (termed "uncoupling" of NOS) in static culture (Thomas et al. 2002; Cai et al. 2003b; Kuzkaya et al. 2003; Landmesser et al. 2003). Previous studies have demonstrated increased NO formation and NOS contribution due to increased $O_2^{\cdot-}$ under H_2O_2-induced oxidative stress (Coyle et al. 2004). We therefore investigated the role of NOS in H_2O_2-induced increases in $O_2^{\cdot-}$ in PAEC under physiologic shear.

The contribution of NOS to H_2O_2-induced increases in $O_2^{\cdot-}$ in PAEC was investigated using N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS activity. L-NAME did not affect basal DHE fluorescence (Figure 4.4). With 1 mmol/L L-NAME treatment, 24.9 ± 8.9% of H_2O_2-exposed PAEC exhibited fluorescent nuclei as compared with 54.6 ± 10.3% of control PAEC, H_2O_2-exposed PAEC (Figure 4.4; n=6, $p<0.05$). These findings suggest that NOS contributes to increased PAEC intracellular $O_2^{\cdot-}$ with exposure to H_2O_2 under shear.

4.4.4 NADPH Oxidase is an Enzymatic Source of $O_2^{\cdot-}$ in PAEC Under Physiologic Shear

Besides NOS, endothelial cells contain various enzymes, such as NADPH oxidase, that are capable of generating $O_2^{\cdot-}$. Moreover, levels of H_2O_2 similar to those used in the present study were shown to activate NADPH oxidase in SMC, leading to increased $O_2^{\cdot-}$ production and cytotoxicity under static conditions (Li et al. 2001). NADPH oxidase has previously been shown to contribute to increased $O_2^{\cdot-}$ under H_2O_2-induced oxidative stress in PAEC in static culture (Coyle et al. 2004). Under physiologic shear, we tested whether treatment with apocynin could block the H_2O_2-induced increase in $O_2^{\cdot-}$ levels in PAEC. When apocynin-treated PAEC were exposed to H_2O_2, there was a significant inhibition of nuclear DHE fluorescence, indicative of reduced intracellular $O_2^{\cdot-}$ levels (Figure 4.4). With apocynin treatment, only 25.2 ± 4.7% of H_2O_2-exposed PAEC exhibited DHE fluorescence (n=4, $p<0.01$ as compared with no apocynin treatment).

These data suggest that NADPH oxidase contributes to increased PAEC $O_2^{\cdot-}$ with exposure to H_2O_2 under shear.

4.4.5 NOS and NADPH Oxidase Co-inhibition Decrease $O_2^{\cdot-}$ Levels in PAEC under Shear

In addition, previous studies by Coyle et al. suggest that NOS and NADPH oxidase contribute to increased $O_2^{\cdot-}$ in PAEC under H_2O_2-induced oxidative stress in static culture (Coyle et al. 2004). Therefore, we probed the effects of the combined contribution of NOS and NADPH oxidase to H_2O_2-induced increases in $O_2^{\cdot-}$ in PAEC using L-NAME and apocynin under shear conditions. Combination of L-NAME and apocynin moderately increased basal DHE fluorescence in the absence of H_2O_2 to 8.7 ± 3.2% from basal levels (2.6 ± 1.7%; n=4, $p<0.05$). However, when L-NAME and apocynin treated PAEC were exposed to H_2O_2, there was a distinct reduction of nuclear DHE fluorescence, indicative of reduced intracellular $O_2^{\cdot-}$ (Figure 4.5). With inhibitor treatment, only 24.3 ± 6.7% of H_2O_2-exposed PAEC exhibited fluorescent nuclei as compared with 70.3 ± 5.2% of vehicle-treated, H_2O_2-exposed PAEC (n=4, $p<0.001$). These findings suggest that NOS and NADPH oxidase inhibition of $O_2^{\cdot-}$ formation in PAEC is additive compared with their individual contributions under physiologic shear. This finding contrasts the non-additive effect observed under static culture.

4.5 Discussion

The current study probes the effects of PAEC exposure to H_2O_2 under static and shearing conditions. Three major conclusions can be drawn from this study: 1) In PAEC, both NOS and NADPH oxidase contribute to increased $O_2^{\cdot-}$ under H_2O_2-induced oxidative stress under physiologic shear; 2) The combined contribution of NOS and NADPH oxidase under static conditions is less then their combined individual effects; 3) The combined contribution of NOS and NADPH oxidase under physiologic shear is synergistic with their individual effects.

Reactive oxygen species (ROS) encompass a number of molecules with unique characteristics (reactivity, half-lives, and cellular targets). H_2O_2, a relatively stable ROS, has attracted considerable interest as a modulator of vascular cell function and viability in physiologic and pathologic states. Endogenous H_2O_2 has been shown to be an important signaler in both smooth muscle cells and endothelial cells with regard to proliferation and apoptosis signaling (Brown et al. 1999; Li et al. 1999; Zanetti et al. 2002). In diseased states, as well as with angiotensin II signaling, H_2O_2 plays a role in the activation of inflammatory genes including the translocation of NF-kB to the nucleus (Griendling et al. 2000a; Griendling et al. 2000b; Pueyo et al. 2000; Taniyama et al. 2003) and increased expression of intercellular adhesion molecule-1 (Bradley et al. 1993; Pueyo et al. 2000).

H_2O_2-induced oxidative stress results in increased $O_2^{\cdot-}$ in smooth muscle cells through NADPH oxidase activation (Li et al. 2000; Li et al. 2001). In a recent study, H_2O_2-induced oxidative stress led to increased $O_2^{\cdot-}$ in endothelial cells under both shear and static culture (Coyle et al. 2004). Under static culture, NADPH oxidase and NOS were identified as contributors to increased $O_2^{\cdot-}$ in PAEC. Previous studies under shear (utilizing a cone and plate viscometer technique) with endothelial cells demonstrated that NADPH oxidase and xanthine oxidase were contributors to increased $O_2^{\cdot-}$ production in response to shear (McNally et al. 2003). Observations in this study suggest that NADPH oxidase and NOS are contributors to increased $O_2^{\cdot-}$ in H_2O_2-induced oxidative stress under shear. This is likely due to increased NOS activity with H_2O_2 exposure as well as stimulation of eNOS activity due to shear stress (Drummond et al. 2000; Davis et al. 2004).

To further probe the contribution of NOS, eNOS was upregulated via adenoviral gene transfer. While no significant change was observed between non-infected and infected PAEC upon exposure to H_2O_2, there was a non-statistical trend suggesting a reduction in $O_2^{\cdot-}$ with eNOS infection under static conditions. This may in part be due to the limited increase in NO production with adenoviral-mediated gene transfer of eNOS.

An increase in peroxynitrite ($ONOO^-$) formation due to increased availability of NO with eNOS upregulation and H_2O_2 stimulation of eNOS activity and increased NO production may also contribute (Thomas et al. 2002; Cai et al. 2003b). Under shear, no statistical change in $O_2^{\cdot -}$ was observed with eNOS upregulation. Further experimentation is necessary under static and shear conditions to determine pathway recruitment in endothelial cells during H_2O_2-induced oxidative stress.

Co-inhibition of NOS and NADPH oxidase was undertaken to probe the combined effects of these pathways under shear and static culture. Observations under static conditions demonstrates a non-additive response of NOS and NADPH oxidase contributions to increased $O_2^{\cdot -}$ under H_2O_2-induced oxidative stress compared with their individual contributions. In contrast, an additive response of NOS and NADPH oxidase contributions to increased $O_2^{\cdot -}$ with H_2O_2-induced oxidative stress was observed under physiologic shear. The non-additive response of NOS and NADPH oxidase under static conditions suggests that NOS uncoupling may rely heavily on NADPH oxidase $O_2^{\cdot -}$ formation and H_2O_2 formation via SOD and $O_2^{\cdot -}$ self-interaction. This is likely as $ONOO^-$ has been shown to react with NOS shifting enzyme production from NO to $O_2^{\cdot -}$. This effect would greatly reduce the effectiveness of the ROS positive feedback mechanism due to decreased $O_2^{\cdot -}$.

The non-additive nature of NOS and NADPH oxidase inhibition may also in part be due to $O_2^{\cdot -}$ reaction kinetics, which suggest a threshold intracellular concentration of NO and $O_2^{\cdot -}$ must be reached prior to the formation of peroxynitrite (Pryor et al. 1995; Quijano et al. 2001; Radi et al. 2001). In part due to the scavenging of $O_2^{\cdot -}$ by SOD, which is thought to be dominant until $O_2^{\cdot -}$ and NO levels are similar, at that time $ONOO^-$ formation becomes the dominant reaction (Pryor et al. 1995; Quijano et al. 2001; Radi et al. 2001). With inhibition of NADPH oxidase, limited $O_2^{\cdot -}$ and H_2O_2 would be available, significantly limiting the formation of $ONOO^-$ as NADPH oxidase is a rapidly recruited

production source of $O_2^{\cdot -}$ (Cai et al. 2000; Wolin et al. 2002; Cai et al. 2003b; Kuzkaya et al. 2003).

This phenomenon is not conserved under shear with dual inhibition resulting in a further reduction of $O_2^{\cdot -}$ compared to the static reduction suggesting a synergistic relationship between co-inhibition of NOS and NADPH oxidase and their individual contributions. Shear stress has also been shown to increase pro-oxidants, including NOS formation of NO and SOD (Malek et al. 1995; Uematsu et al. 1995; Corson et al. 1996; Dimmeler et al. 1999; Woodman et al. 1999). This shift in expression may contribute to the additive response associated with decreased $O_2^{\cdot -}$ under shear; though further experimentation is necessary to further elucidate the pathway changes observed with PAEC exposure to shear.

The suggestion of a non-additive relationship under static culture suggests a complex pathway system, where modeling will be of use. This study also demonstrates the importance of shear testing, as an additive relationship was observed in PAEC under shear. At this time, further experiments are needed to probe additional sources of $O_2^{\cdot -}$ under H_2O_2-induced oxidative stress under both shear and static conditions. The overall goal of these studies is to provide a possible model for disease development within the vasculature. This is further supported by studies suggesting increased local levels of hydrogen peroxide upwards of 100 μmol/L at inflammatory sites (Halliwell et al. 2000); this could have important implications for atherosclerosis, an inflammatory disease.

4.6 Acknowledgements

The authors would like to acknowledge Mr. Gregory Leon, Mr. John D. McRae, and Mr. Luis Martinez for technical assistance and the University of Iowa Biosciences Initiative for funding.

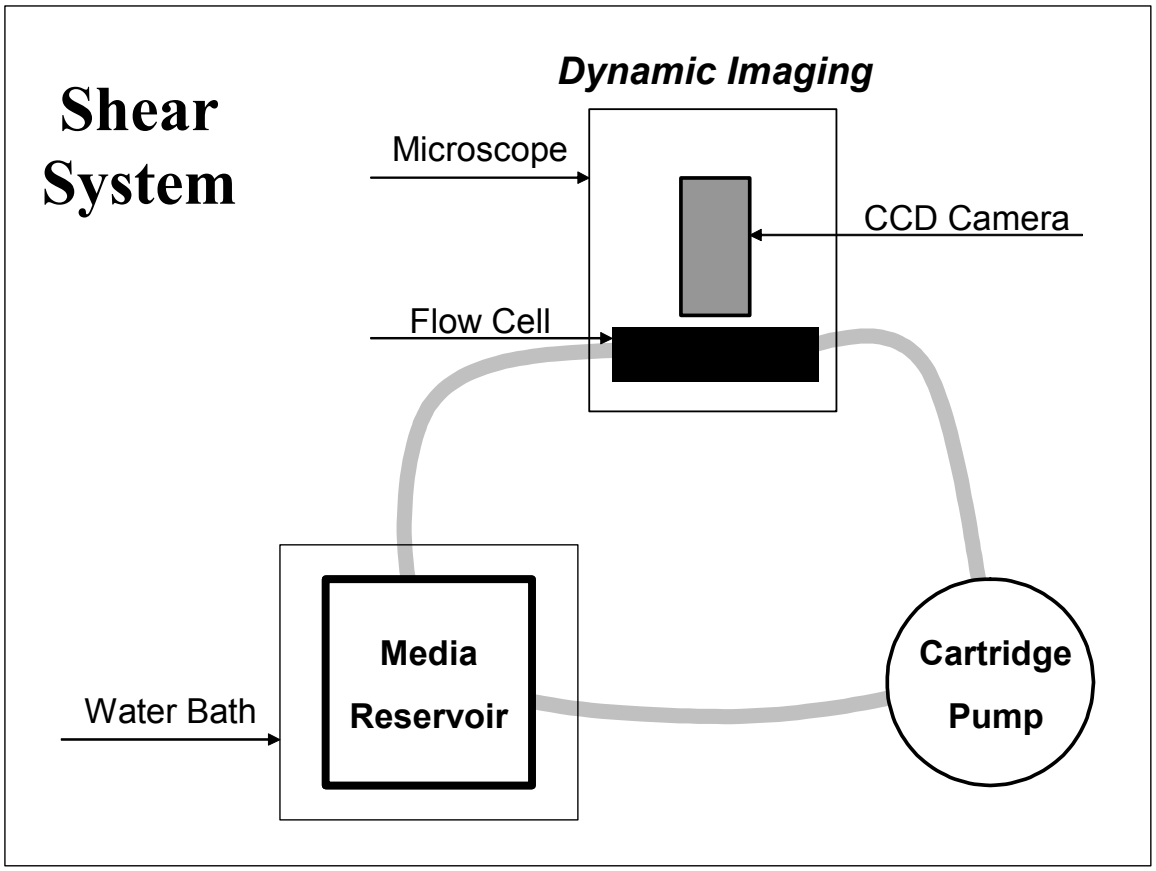

Figure 4.1: Shear System Schematic. Schematic of the shear system, emphasizing the three major components. This includes the peripheral devices, the cartridge pump, and the media reservoir maintained at 37°C in a water bath. The primary component of the system, the Immunetics flow cell, is designed for laminar flow across the cell monolayer. A dynamic imaging system consisting of a Zeiss S100 Inverted Microscope with fluorescence and a Q-Imaging Retiga 1300 CCD camera were also incorporated.

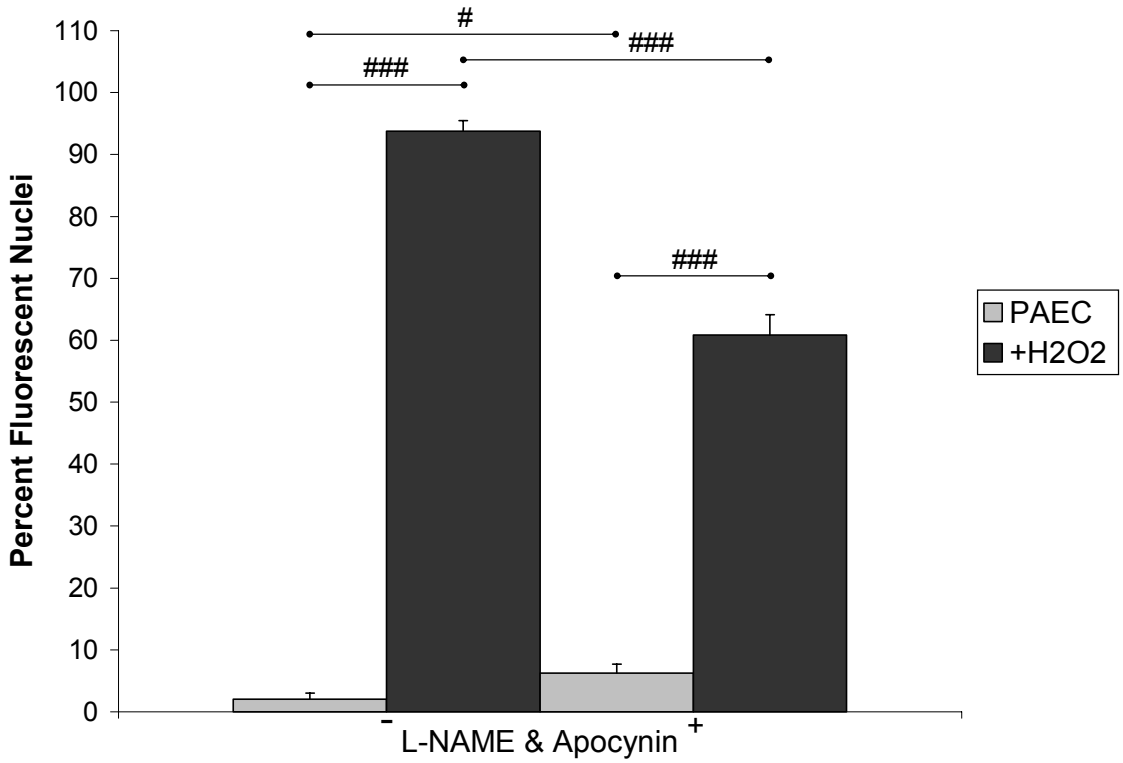

Figure 4.2: Co-inhibition of L-NAME and Apocynin Reduce Hydrogen Peroxide Induced $O_2^{\cdot-}$. PAEC under static conditions were exposed to 0 (control) or 60 µmol/L H_2O_2 and 0 (control) or 1 mmol/L L-NAME (NOS inhibitor) and 250 µmol/L apocynin (NADPH oxidase inhibitor) for 1.5 h, after which intracellular $O_2^{\cdot-}$ was examined using fluorescent microscopy with DHE. Data are expressed as mean ± SEM with (###p<0.001 or ##p<0.01 or #p< 0.05).

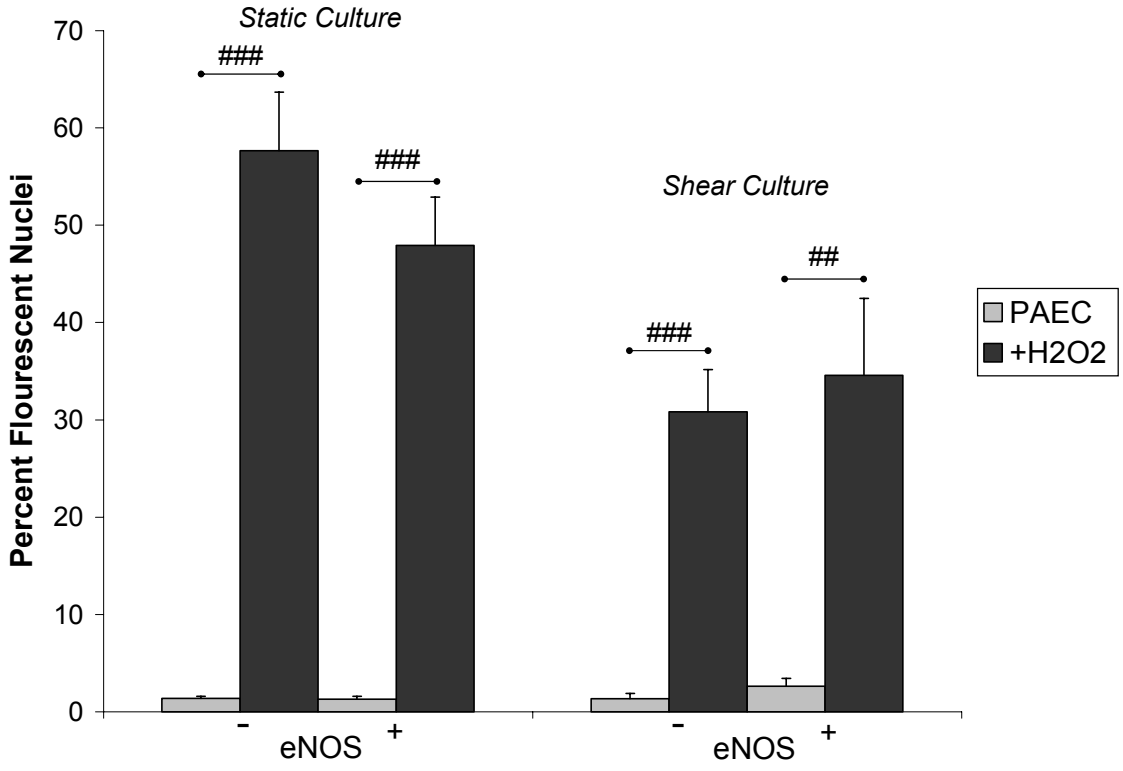

Figure 4.3: Transfer of Human eNOS Gene Does Not Reduce Hydrogen Peroxide Induced $O_2^{\cdot-}$ under Static and Shear Conditions. PAEC under static conditions were exposed to 0 (control) or 60 µmol/L H_2O_2 for 1.5 h and PAEC under shear were exposed to 0 (control) or 100 µmol/L H_2O_2 for 1.5 h, after which intracellular $O_2^{\cdot-}$ was examined using fluorescent microscopy with DHE. Prior to experimentation, some cells were infected with adenoviral vectors expressing eNOS. Data are expressed as mean ± SEM with ($p>0.05$) or (###$p<0.001$ or ##$p<0.01$).

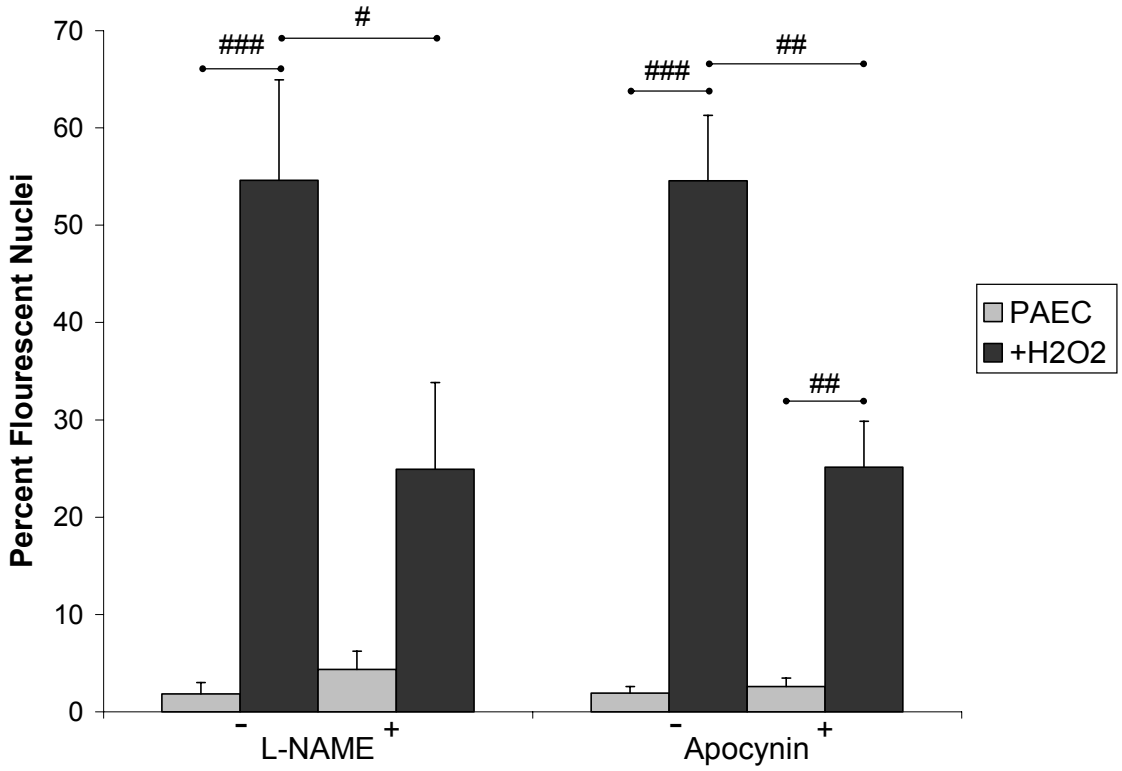

Figure 4.4: L-NAME and Apocynin Reduced Hydrogen Peroxide Induced $O_2^{\cdot-}$ under Shear. PAEC under shear conditions were exposed to 0 (control) or 100 µmol/L H_2O_2 and 0 (control) or 1 mmol/L L-NAME (NOS inhibitor) or 250 µmol/L apocynin (NADPH oxidase inhibitor) for 1.5 h, after which intracellular $O_2^{\cdot-}$ was examined using fluorescent microscopy with DHE. Data are expressed as mean ± SEM with (###$p<0.001$ or ##$p<0.01$ or #$p< 0.05$).

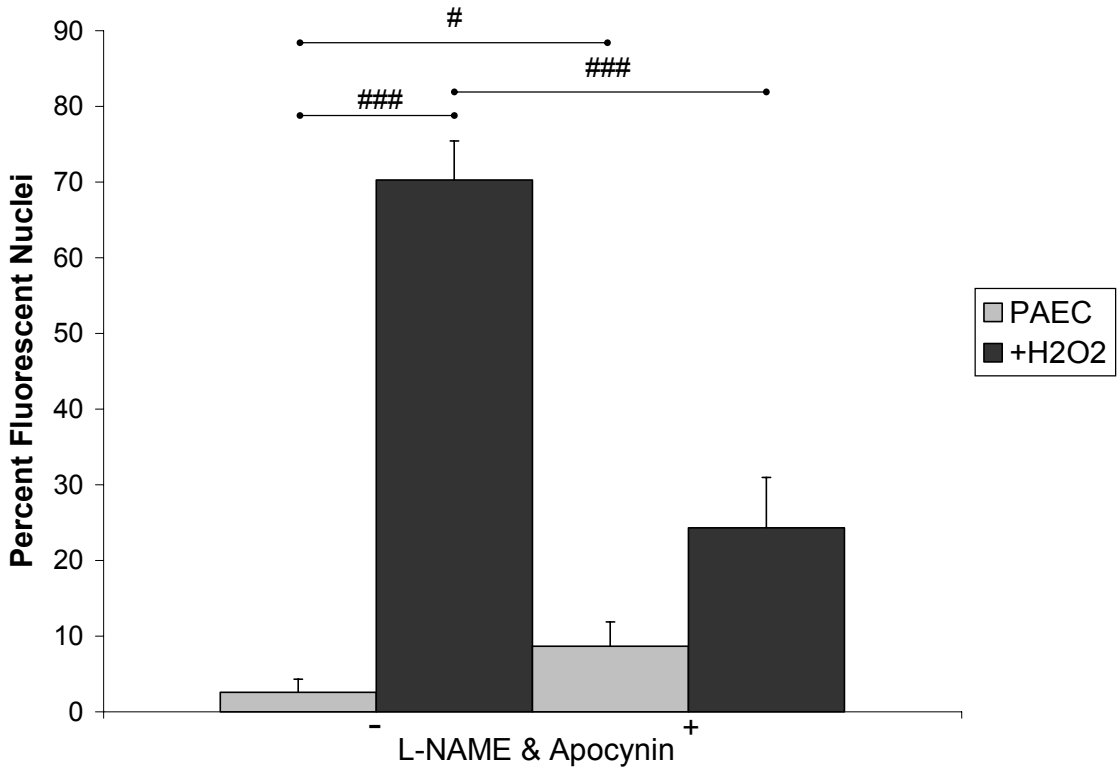

Figure 4.5: Co-inhibition of L-NAME and Apocynin Reduced Hydrogen Peroxide Induced $O_2^{\cdot-}$ under Physiologic Shear. PAEC under shear conditions (8.2 dynes/cm^2) were exposed to 0 (control) or 60 µmol/L H_2O_2 and 0 (control) or 1 mmol/L L-NAME (NOS inhibitor) and 250 µmol/L apocynin (NADPH oxidase inhibitor) for 1.5 h, after which intracellular $O_2^{\cdot-}$ was examined using fluorescent microscopy with DHE. Data are expressed as mean ± SEM with (###p<0.001 or ##p<0.01 or #p< 0.05).

CHAPTER 5
SUMMARY AND CONCLUSIONS

Research into the early stages of cardiovascular disease is a growing need as the prevalence of cardiovascular disease continues to increase world-wide. This research also correlates with the search for new therapy development and early markers of cardiovascular disease. This study was designed to initiate the examination of the suitability of H_2O_2-induced oxidative stress to simulate an oxidative environment similar to cardiovascular disease. Then using this simulated environment, $O_2^{\cdot-}$ formation was probed with exposure to H_2O_2 and enzymatic sources of $O_2^{\cdot-}$ were also probed.

This model was considered due to previous studies that have suggested increased reactive oxygen species (ROS) associated with cardiovascular disease. H_2O_2-induced oxidative stress was also utilized as previous experimentation with vascular smooth muscle cells demonstrated increased smooth muscle cell $O_2^{\cdot-}$ and increased cytotoxicity with H_2O_2 exposure. There is also evidence to suggest a role for ROS in the initial phases of vascular disease development. This is especially true for atherosclerosis due to its inflammatory nature. Increased ROS have been linked with an increase in expression of inflammatory proteins and endothelial cell inflammatory surface receptors. Increased local extracellular levels of H_2O_2 have also been associated with inflammation and several studies have suggested increased systemic levels of H_2O_2 with cardiovascular disease.

Three projects were designed to probe the effects of H_2O_2-induced oxidative stress in endothelial cells. The first project probed PAEC $O_2^{\cdot-}$ in response to H_2O_2 exposure. Endothelial cell $O_2^{\cdot-}$ significantly increased in the presence of H_2O_2 compared to control. The increase in $O_2^{\cdot-}$ was attenuated by MnSOD adenoviral-mediated gene transfer and Tiron. Then, enzymatic pathways associated with $O_2^{\cdot-}$ production were probed to determine the sources of $O_2^{\cdot-}$. NOS and NADPH oxidase were found to be

contributors to increased endothelial cell $O_2^{\cdot-}$. H_2O_2-induced oxidative stress was also shown to increase endothelial cell cytotoxicity, which was limited by exposure to L-NAME, Tiron, or MnSOD adenoviral mediated gene transfer.

These data were significant as they documented the ability of H_2O_2 to increase $O_2^{\cdot-}$ in endothelial cells and demonstrated increased endothelial cell cytotoxicity due to $O_2^{\cdot-}$. The inhibition studies then isolated two enzymatic pathways that contributed to increased $O_2^{\cdot-}$in endothelial cells exposed to H_2O_2. One possible pathway that could be responsible would be c-Src activation of NADPH oxidase $O_2^{\cdot-}$ production. Stimulation of c-Src would contribute and increase intracellular $O_2^{\cdot-}$. Increased NO levels have been shown with H_2O_2 stimulation of eNOS. With formation of both $O_2^{\cdot-}$ and NO, this could result in peroxynitrite formation and overall increases in ROS (Figure 5.1). After demonstrating increased $O_2^{\cdot-}$ and two enzymatic sources under static conditions, the next step was to examine endothelial cell function with exposure to H_2O_2 under shear conditions as endothelial cells are exposed to shear *in vivo*.

The second project consisted of the development of a shear system to dynamically probe PAEC $O_2^{\cdot-}$ under shear with the fluorescent probe dihydroethidium. Then, the effects of H_2O_2-induced oxidative stress in endothelial cells under laminar shear were probed utilizing the developed shear system. Under shear, endothelial cell intracellular $O_2^{\cdot-}$ was significantly increased in the presence of H_2O_2 compared to control. The increase in $O_2^{\cdot-}$ was again attenuated by MnSOD adenoviral-mediated gene transfer and Tiron. eNOS adenoviral-mediated gene transfer had no significant effect on $O_2^{\cdot-}$, though this may be due to the limited increase in NO production observed with eNOS upregulation. NOS and NADPH oxidase were probed as possible $O_2^{\cdot-}$ enzymatic sources and were found to contribute to increased endothelial cell $O_2^{\cdot-}$ under shear in the presence of H_2O_2. H_2O_2-induced oxidative stress was also shown to reduce endothelial cell attachment under shear and therefore viability, which was limited by exposure to L-NAME, apocynin, or Tiron.

The third project was designed to begin to probe the interrelationship between the multiple pathways of ROS production, eventually leading to a model of endothelial cell response to H_2O_2-induced oxidative stress. The first step examined the combined contribution of NOS and NADPH oxidase to $O_2^{\cdot-}$ under both shear and static conditions.

Co-inhibition demonstrated a non-additive relationship between NADPH oxidase and NOS inhibition as the percent fluorescent cells with co-inhibition under static culture were not statistically different then either of the individual inhibitors. This is possibly due to the reliance of one of the pathways on one another for the given effects. Previous studies would suggest NOS reliance upon NADPH oxidase (see Figure 5.1). This is plausible as NOS requires an alternate source of $O_2^{\cdot-}$ prior to uncoupling, uncoupling requires the formation of peroxynitrite. c-Src is an important protein in the sequence of NADPH oxidase activation, its activity is increased by H_2O_2. Elevated levels of H_2O_2 may contribute to significant $O_2^{\cdot-}$ production via NADPH oxidase. This suggests a possible signaling pathway involving increased c-Src mediated phosphorylation of $p47^{phox}$ and increased NADPH oxidase $O_2^{\cdot-}$ output allowing for $ONOO^-$ formation and NOS uncoupling (see Figure 5.2). Further experiments are necessary to determine if this type of response is observed, as there are multiple alternative sources of $O_2^{\cdot-}$ within endothelial cells.

Under shear, co-inhibition demonstrated a synergistic relationship between NADPH oxidase and NOS inhibition as the co-inhibition was statistically different then either of the individual inhibitors. This suggests a different mechanism then was observed under static conditions is acting under shear. The upregulation of both pro-oxidants and anti-oxidants (NO and SOD) by shear stress may be a contributing factor; additional experimentation is necessary to further elucidate the change in pathway interactions under shear.

Overall this study suggests that H_2O_2-induced oxidative stress may be a reasonable mimic for cardiovascular disease. Additional studies are necessary to further

isolate the signaling pathways responsible for the effects of H_2O_2 and increased $O_2^{\cdot-}$ to determine if peroxynitrite is the agent responsible for NOS uncoupling.

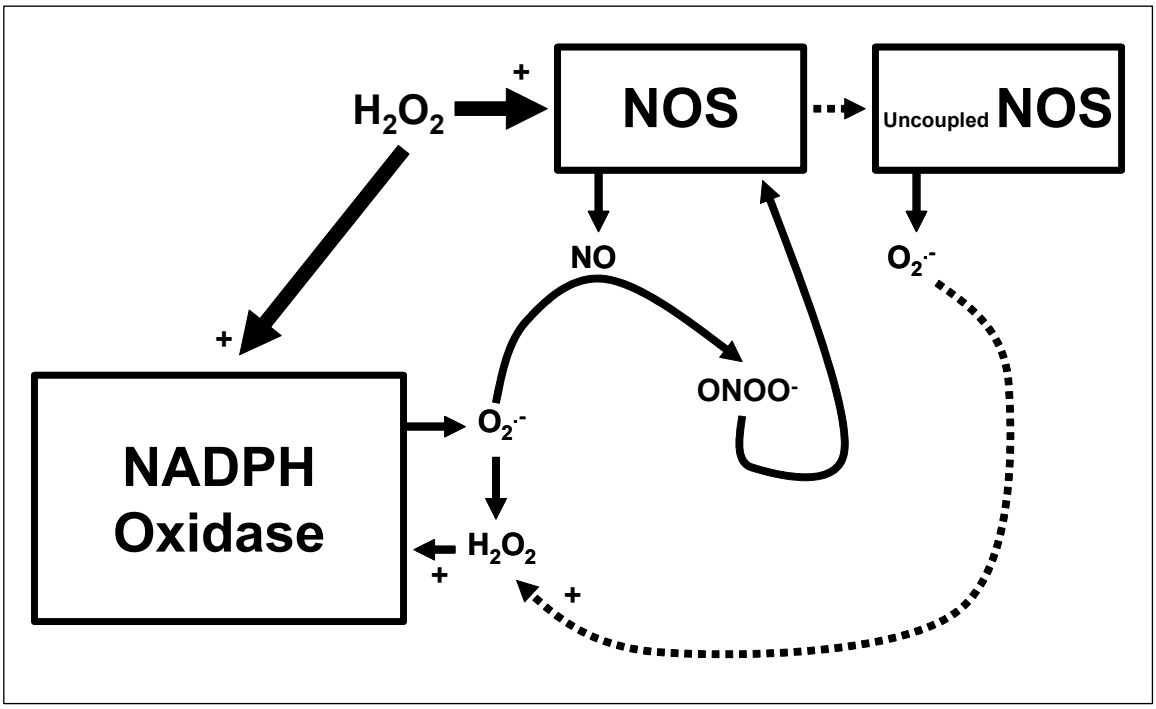

Figure 5.1: Possible Interactions Between NOS and NADPH Oxidase Resulting in ROS Production. This figure summarizes the possible interactions between NOS and NADPH oxidase. This demonstrates the possible reliance upon NADPH Oxidase for $O_2^{\cdot-}$ to lead to uncoupled NOS via $ONOO^-$ with H_2O_2 stimulus.

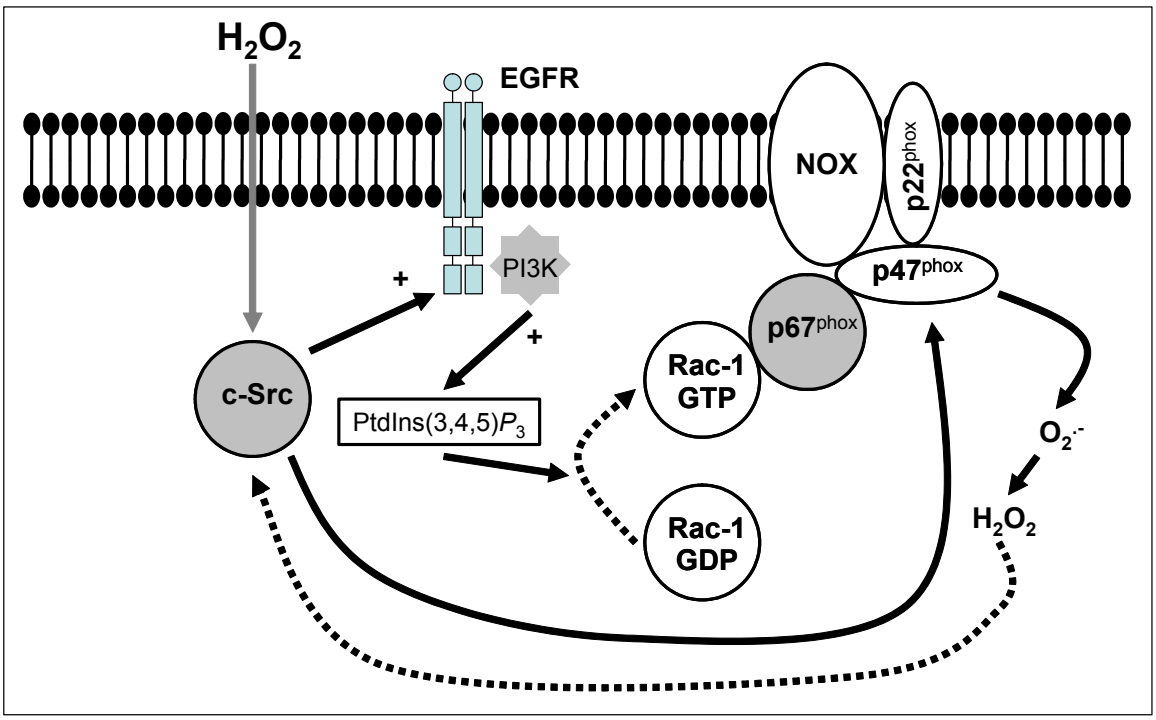

Figure 5.2: Possible Interactions Between NOS and NADPH Oxidase: NADPH Oxidase Signaling. This figure summarizes the possible interactions with H_2O_2 stimulation of $O_2^{\cdot-}$ production. It is possible that H_2O_2 directly activated NADPH oxidase via c-SRC stimulation leading to a feedback loop and significant $O_2^{\cdot-}$ production via NADPH oxidase. This is a possible mechanism that may allow for NOS uncoupling.

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