Mechanisms of Endothelin-1 induced Reactive Oxygen Species Production in Vascular Adventitial Fibroblasts

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A thesis submitted for completion of the Master of Science Degree (Health Science)

in the

Faculty of Applied Health Sciences
Brock University
St. Catharines, ON

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Abstract

With the relationship between endothelin-1 (ET-1) stimulation and reactive oxygen species (ROS) production unknown in adventitial fibroblasts, I examined the ROS response to ET-1 and angiotensin (Ang II).

ET-1-induced ROS peaked following 4 hrs of ET-1 stimulation and was inhibited by an ET$_A$ receptor antagonist (BQ 123, 1 μM) an extracellular signal-regulated kinase (ERK) 1/2 inhibitor (PD98059, 10 μM), and by both a specific, apocynin (10 μM), and non-specific, diphenyleneiodonium (10 μM), NAD(P)H oxidase inhibitor. NOX2 knockout fibroblasts did not produce an ET-1 induced change in ROS levels.

Ang II treatment increased ROS levels in a biphasic manner, with the second peak occurring 6 hrs following stimulation. The secondary phase of Ang II induced ROS was inhibited by an AT$_1$ receptor antagonist, Losartan (100 μM) and BQ 123.

In conclusion, ET-1 induces ROS production primarily through an ET$_A$-ERK1/2 NOX2 pathway, additionally, Ang II-induced ROS production also involves an ET$_A$ pathway.
Acknowledgements

I would like to thank the members of the vascular biology lab at Brock University. In particular, I would like to thank Shenjgun An, Lili Ding, Ryan Boyd and Andrea Forgione for their patience and cooperation in aiding me complete this thesis. Additionally, I would like to thank Dr. Brian Roy and Dr. Evanlegia Tsiani for the use of their laboratories and equipment.

I would also like to thank Dr. David Lambeth of Emory University for supplying the NOX4 antibody.
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Abbreviations

ACE: angiotensin converting enzyme
AFU: arbitrary fluorescence unit
α-SM-actin: α-smooth muscle actin
AM: adrenomedullin
Ang II: angiotensin II
ANOVA: analysis of variance
AP-1: activator protein-1
ATP: adenosine triphosphate
bFGF: basic fibroblast growth factor
BH_4: tetrahydrobiopterin
BP: blood pressure
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<td>endothelial nitric oxide synthase</td>
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<td>ERK</td>
<td>extracellular-response kinase</td>
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<td>FAD</td>
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Introduction / Literature Review

I. Adventitia

The structure of a blood vessel can be described as having three distinct layers, the *tunica intima*, *tunica media* and *tunica adventitia*. Endothelial cells (EC) and vascular smooth muscle cells (VSMC) of the intima and media respectively have received considerable scientific attention while the adventitia has essentially been overlooked (Sartore et al., 2001). While the *tunica intima* and *tunica media* are mainly composed of ECs and smooth muscle cells (SMC) respectively, the primary cell type of the *tunica adventitia* is the fibroblast. Significant levels of fibroblast products collagen and elastin are found in the *tunica adventitia* region, along with an assortment of other cell types, such as mast cells, macrophages and ganglionic cells (Rey & Pagano, 2002). More recent research has expanded on the classical functions of adventitia and has demonstrated the ability of the adventitia to be a significant producer of growth factors and of adventitial fibroblasts to phenotypically differentiate into a more active and mobile myofibroblast form.

1.1. Role in extracellular matrix protein expression

In the past, the adventitia was only thought to have two classical functions. The first role is scaffolding for both sympathetic nerve endings and the vasa vasorum so that they are able to innervate the vessel (Rey et al., 2002). The other classical role of the adventitial fibroblast is the production of significant amounts of extracellular matrix (ECM) proteins, particularly collagen and elastin, as these two components comprise a considerable amount of the *tunica adventitia* (Rey et al., 2002). Although physiological levels of ECM are required for proper vascular function, the accumulation of interstitial
collagen is associated with vascular disease (Intengan & Schiffrin, 2001; Park & Schiffrin, 2001). Excessive deposition of collagen and ECM proteins due to systemic hypertension occurs throughout the vessel with initial build up taking place in the adventitial region of arterioles (Nicoletti & Michel, 1999).

Increased production of collagen has been demonstrated in adventitial fibroblasts due to stimulation with several mitogens, including angiotensin II (Ang II) (An, Boyd, Wang, Qiu, & Wang, 2006) and endothelin-1 (ET-1) (An et al., 2007). The increased synthesis of procollagen, the precursor of collagen has been observed in cardiac fibroblasts by several other substances and forces, including mechanical load (Carver, Nagpal, Nachtigal, Borg, & Terracio, 1991), platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and insulin-like growth factor-1 (IGF-1) (Bishop & Lindahl, 1999). Transforming growth factor-β (TGF-β) has also demonstrated the ability to increase procollagen production in both cardiac fibroblasts (Bishop et al., 1999) and in interstitial fibroblasts of the pulmonary vascular system (Lindahl et al., 2002). Additionally, TGF-β, PDGF and bFGF stimulation lead to a decrease in collagen degradation (Bishop et al., 1999).

The production of another ECM protein, fibronectin, is also proving to be of significance as it is required for the conversion of fibroblasts to activated fibroblasts, or myofibroblasts (Gabbiani, 2003) and the ensuing migration that has been demonstrated to occur following injury (Greiling & Clark, 1997), particularly during neointimal formation.

The classical functions of the adventitia, and its primary cell type, the adventitial fibroblast, include the innervation of nerve endings and production of ECM proteins.
These characteristics are certainly of importance in the study of vascular disease, however, the premise that these are the only functions of the adventitia are outdated.

1.2. Role in growth factor expression

In addition to the two classic functions of adventitial fibroblasts, one area in which adventitial fibroblasts have the ability to influence the entire vessel is through the release of paracrine factors. At present, this area of research remains primarily unexplored as few studies have examined adventitial fibroblasts in an in vitro setting. The key mitogens that are known to be produced by adventitial fibroblasts and function in an autocrine manner are ET-1 (An et al., 2007) and TGF-β (Long, Henrich, & Simpson, 1991). Studies conducted on pulmonary artery adventitial fibroblasts have also shown that PDGF, insulin-like growth factor-1 (IGF-1) (Stenmark, Davie, Frid, Gerasimovskaya, & Das, 2006), and adenosine triphosphate (ATP) can be released extracellularly to promote replication in the tunica adventitia (Gerasimovskaya et al., 2002). A further growth factor that has been shown to be produced by cardiac and mammary fibroblasts is basic fibroblast growth factor (bFGF) or FGF-2 (Bishop et al., 1999; Delehedde et al., 2000).

In vivo studies have demonstrated autocrine functions of these peptides. The difficulty for researchers is trying to assess the function of the peptide released from each of the three layers of the vessel. Thus, although these peptides have been demonstrated to be released in the adventitia, it is unclear if they may also have a paracrine role within the vessel.

In the same way that it is difficult to determine the role of a growth factor released from the adventitial on the entire vessel, the same issue occurs in trying to determine the
significance of production of growth factors that are synthesized at all three level of the vessel. This obstacle is apparent when examining peptides such as PDGF, IGF-1, TGF-β and bFGF as all are produced in all regions of the vessel (Bishop et al., 1999). This issue has been illustrated in several studies that utilized immunohistochemistry to examine complete vessels during the process of angiogenesis. For example, Cai et al. (2003) examined urokinase-type plasminogen (uPA), plasminogen activator inhibitor (PAI)-1, and bFGF expression during coronary arteriogenesis via a chronic occlusion in the left coronary vessel in canine hearts. The results that where attained through immunohistochemistry of the vessel were conclusive in demonstrating that levels of these peptides increase in the adventitia following occlusion (Cai et al., 2003). The dilemma is trying to establish the origin of these peptides, as for example bFGF has been shown to be produced by ECs via TGF-β stimulation, (Ferrari et al., 2006) a growth factor that is potentially elevated in this model.

1.3. Role in neointima formation and pulmonary hypertension

Two models or areas of study in which the adventitial region has received attention include neointima formation and pulmonary hypertension due to hypoxia. Although several adventitial fibroblast-derived substances may be involved in neointima formation, a majority of the research in this field revolves around the conversion of adventitial fibroblasts to "active" myofibroblasts. In vivo work with inhibitors specifically inhibiting adventitial fibroblast function has clearly concluded that adventitial fibroblasts play a significant role in neointimal formation (Dourron et al., 2005; Weaver et al., 2006). Controversy still remains as to the exact mechanisms by which adventitial fibroblasts mediate this formation. Debate continues as to whether neointimal growth is
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primarily due to migration of myofibroblasts or due to production of paracrine factors from myofibroblasts (Maeng et al., 2003; Li, Chen, Oparil, Chen, & Thompson, 2000; Li et al., 2002b; Scott et al., 1996; Shi et al., 1996; Siow, Mallawaarachchi, & Weissberg, 2003).

Similarly, hypoxia-induced pulmonary hypertension and models replicating this condition have increased interest in the function of pulmonary adventitial fibroblasts. This interest was sparked with research which demonstrated that the earliest and most striking structural changes observed in hypoxic animal models occurred in the adventitia. The modifications included hyperplasia of adventitial fibroblasts leading to adventitial thickening and increased stiffness in the pulmonary artery. Although media and intimal remodelling does occur subsequent to adventitial remodelling, both are dependent on the initial remodelling of the adventitial (Stenmark, Frid, Nemenoff, Dempsey, & Das, 1999; Durmowicz & Stenmark, 1999). Subsequent research has also demonstrated that under hypoxic conditions, hypoxia-inducible factor-1α (HIF-1α) becomes stabilized in pulmonary adventitial fibroblasts, leading to upregulation of several genes necessary for survival (Krick et al., 2005). One angiogenic factor that is produced under these conditions is vascular endothelial growth factor (VEGF) (Krick et al., 2005). VEGF is involved in the expansion of the vasa vasorum, the network of blood vessels imbedded in the adventitia, helping supply O₂ and nutrients to the increase density of cells.

Substantial attention has been given to this characteristic of the adventitia, not just in hypoxic-induced pulmonary hypertension, but also in intimal hyperplasia, as blocking the supply of nutrients could inhibit hyperplasia (Khurana et al., 2004). Having stated and discussed the characteristics of the pulmonary adventitial fibroblasts during hypoxic
conditions, it is worth noting that although systemic and pulmonary fibroblasts may have very similar capabilities in many circumstances, they are still unique cell types. In hypoxic conditions, pulmonary adventitial fibroblasts proliferate where as systemic adventitial fibroblasts from either aorta or mesenteric vasculature do not proliferate (Welsh, Scott, Plevin, Wadsworth, & Peacock, 1998; Welsh, Peacock, MacLean, & Harnett, 2001) or may even have an increased level of apoptosis (Krick et al., 2005). It is due to this distinctiveness among fibroblasts of assorted origins that it is necessary to try to focus on the observations made while examining adventitial fibroblasts of the systemic circulation, as all fibroblasts are not equal and responses between differing fibroblasts communities may be distinct. Having made this distinction, although the research regarding hypoxia-induced pulmonary hypertension is unique to the pulmonary system, the mechanism involved in neointimal formation and hypoxia-induced pulmonary hypertension demonstrate that adventitial fibroblasts can have a significant role in pathologies affecting circulation.

1.4. Adventitial fibroblasts versus myofibroblasts

Despite the understanding that the majority of the adventitia is composed of fibroblasts, recent research in the vascular field has examined the differences in function, and possible pathological consequences of the differentiation that can occur between adventitial, or so-called inactive fibroblasts and active myofibroblasts, particularly in neointimal injury. A hallmark adventitial sign to neointimal injury is the conversion of adventitial fibroblasts to myofibroblasts and the subsequent migration to site of injury in the intima layer (Li et al., 2000; Li et al., 2002b; Scott et al., 1996; Shi et al., 1996; Siow et al., 2003).
One key alteration that occurs during this conversion is the production of $\alpha$-smooth muscle (SM) actin, one of the earliest proteins formed in SM differentiation (Owens, 1995). It has been hypothesized that all three cell types, fibroblasts, myofibroblasts and SM cells are derived from the same progenitor cells, and that the expression of the phenotypic markers, such $\alpha$-SM actin and myosin heavy chain (MHC), can allow classification of these cell types. Using this classification system, fibroblasts express neither $\alpha$-SM actin nor MHC, myofibroblasts express only $\alpha$-SM actin and SM cells generate both markers (Sartore et al., 2001).

It has been speculated that myofibroblasts may be of consequence in both atherosclerosis and hypertension due to their increased rate of proliferation as well as augmented production of ECM proteins, particularly collagen (Sun & Weber, 1996; Shen et al., 2006; Maiellaro & Taylor, 2007).

Another hypothesis is the possibility of myofibroblasts having an increased ability to release a larger number and quantity of growth factors and cytokines (Plekhanova et al., 2006). This hypothesis is supported by data from idiopathic pulmonary fibrosis patients. In the study conducted by Shahar et al. (1999), fibroblast cell cultures from human lung biopsies demonstrated an increased level of $\alpha$-SM actin expression in idiopathic pulmonary fibrosis patients along with a corresponding increase in the release of ET-1 compared with controls (Shahar et al., 1999). Although the differences between adventitial fibroblasts and myofibroblasts may appear only small, the consequences of these differences may have significant ramifications in the pathophysiology of several cardiovascular diseases.
Investigation regarding the conversion of adventitial fibroblasts to myofibroblasts outside of neointimal injury has only recently elicited interest in the cardiovascular community. Recent enquiries have examined various stimuli that have the ability to mediate this conversion, and the signal transduction pathways required for these differentiation. Shi-Wen et al. (2004) demonstrated that ET-1, the most potent known vasoconstrictor, has the capacity to cause differentiation of lung adventitial fibroblasts to myofibroblasts through the endothelin-type A (ET$_A$) receptor via a rac/phosphoinositide 3-kinase/Akt dependent pathway. Similarly, the vasoconstrictor Ang II has also demonstrated this capacity in rat adventitial fibroblasts, however, the reactive oxygen species (ROS) producing nicotinamide adenine dinucleotide phosphate – oxidase (NAD(P)H) was implicated as the mediator of this conversion (Shen et al., 2006).

Finally, the vasoconstrictor/growth factor TGF-β has also been examined in cardiac fibroblasts and rat adventitial fibroblasts (Cucoranu et al., 2005; Guo et al., 2006). Within both forms of fibroblasts, NAD(P)H oxidase was implicated as a mediator of this conversion as Guo et al. (2006) demonstrated an upregulation of NAD(P)H oxidase genes during the differentiation process where as Cucoranu et al. (2005) was able to inhibit the differentiation through inhibition of NAD(P)H oxidase. Prior research supports this theory as treatment with an endogenous antioxidant, adrenomedullin (AM), also inhibited this conversion in vivo (Tsuruda et al., 2005).

In summary, these results reveal that the differentiation of adventitial fibroblasts to myofibroblasts is possible through several stimuli; however the presence of a redox-sensitive pathway in three of the studies may suggest a reliance on ROS generation for differentiation to occur.
II. Reactive Oxygen Species

The role of excessive ROS in the pathogenesis in several cardiovascular diseases has been well accepted. Recently, ROS have been considered as a vital part of normal cell signaling and regulation (Griendling & Ushio-Fukai, 2000). As an example, increasing evidence is supporting a role for the ability of ROS, specifically \( \text{H}_2\text{O}_2 \), to reversibly modify cysteine residues thus controlling the activity tyrosine phosphatases and peroxiredoxins (Salmeen & Barford, 2005). ROS have also been suggested in the conversion of adventitial fibroblasts to myofibroblasts. The following overview will examine the ROS family and the vascular enzymes known to be generators of ROS.

II.1. Reactive Oxygen Species Family

The major forms of ROS include superoxide \( (\cdot\text{O}_2^-) \), hydrogen peroxide \( (\text{H}_2\text{O}_2) \), hydroxyl radical \( (\cdot\text{OH}) \), nitric oxide \( (\cdot\text{NO}) \) and peroxynitrite \( (\text{ONOO}^-) \). Until recently, these ROS were thought solely to be injurious cellular by-products of oxidative metabolism. Not only has it become accepted that ROS are involved in normal cell signaling but also that overproduction of ROS, known as oxidative stress, is significantly involved in numerous cardiovascular diseases, including hypertension.

The ROS in the vasculature that has been studied extensively is superoxide. Formed through a univalent reduction of oxygen, superoxide is regarded as such an important ROS due to its ability to initiate vascular effects itself, or, through its degradation via antioxidant mechanisms, superoxide has the potential to produce \( \text{H}_2\text{O}_2 \), \( \cdot\text{OH} \), or \( \text{ONOO}^- \). If not used as an oxidizing agent, the reduction of superoxide to \( \text{H}_2\text{O}_2 \) can happen either spontaneously or enzymatically through superoxide dismutase (SOD). Subsequently, \( \text{H}_2\text{O}_2 \) is converted to water by catalase or glutathione peroxidase. \( \text{H}_2\text{O}_2 \), a
much more stable ROS, also has the ability to be detrimental itself or react with transition metals, particularly iron (Fridovich, 1997), and convert to \( \cdot \text{OH}^{\cdot} \) another highly reactive ROS. One effect of superoxide overproduction is the reaction that occurs with \( \cdot \text{NO}^{\cdot} \), a potent vasodilator. This reaction of excess superoxide with \( \cdot \text{NO}^{\cdot} \) leads to a decrease in \( \cdot \text{NO}^{\cdot} \) bioavailability. Additionally, the superoxide + \( \cdot \text{NO}^{\cdot} \) reaction produces \( \text{ONOO}^{\cdot} \), an even more powerful oxidizing agent than superoxide (Taniyama & Griendling, 2003).

One significant difference between \( \text{H}_2\text{O}_2 \) and other ROS, such as superoxide, \( \cdot \text{OH}^{\cdot} \) and \( \text{ONOO}^{\cdot} \), is \( \text{H}_2\text{O}_2 \)'s ability to flow freely through cell membranes due to its lipid solubility. This characteristic of \( \text{H}_2\text{O}_2 \) theoretically allows it much more freedom within the vessel wall. Other ROS, such as superoxide require anion channels to flow through cell membranes, significantly impeding its movement (Touyz & Schiffrin, 2004a). As previously discussed, ROS play a role in maintaining proper vascular function, however, once antioxidant defences become overwhelmed, oxidative stress ensues, contributing to numerous cardiovascular diseases, such as; hypertension, diabetes, atherosclerosis, ischemic-reperfusion injury, ischemic heart disease and congestive cardiac failure (Landmesser & Harrison, 2001).

II.2. Composition of NAD(P)H oxidase

Vascular ROS can be produced via several different enzymatic reactions, however, the predominate and most scrutinized ROS producing enzyme, NAD(P)H oxidase, is responsible for a majority of ROS production within the vessel wall (Touyz et al., 2004a).

The enzyme NAD(P)H oxidase was not originally recognized for it's role in the cardiovascular system, but the immune system, as it functions as the primary source of
ROS in phagocytes (Babior, Lambeth, & Nauseef, 2002). In these cell types, as well as within the non-phagocytic cells with NAD(P)H oxidase, the one electron reduction of oxygen occurs with NAD(P)H as the electron donor.

The reduction of oxygen via the classical phagocytic NAD(P)H oxidase occurs through the assembly of five principal subunits, \( p47^{\text{phox}} \), \( p67^{\text{phox}} \), \( p40^{\text{phox}} \), \( p22^{\text{phox}} \) and \( gp91^{\text{phox}} \) (phox for PHagocyte OXidase) (Touyz et al., 2004a). The multi-unit enzyme is comprised of 3 cytosolic subunits, \( p47^{\text{phox}} \), \( p67^{\text{phox}} \) and \( p40^{\text{phox}} \), with \( p22^{\text{phox}} \) and the \( gp91^{\text{phox}} \) subunit being membrane bound and bound together in a heterodimeric flavoprotein complex known as cytochrome b558. Additionally, small G protein Rac 1 or 2, depending on cell type, and Rap1A (Babior et al., 2002).

Upon activation, \( p47^{\text{phox}} \) becomes phosphorylated and a complex is formed between all cytosolic subunits. The cytosolic complex then migrates to the membrane to associate with cytochrome b558. The final step involves the GTPase Rac first interacting with the \( gp91^{\text{phox}} \) subunit (Diebold & Bokoch, 2001), then the \( p67^{\text{phox}} \) subunit (Lapouge et al., 2000), at which point the enzyme becomes active. The importance of \( p47^{\text{phox}} \) and \( p67^{\text{phox}} \) phosphorylation to activation of cytochrome b558 has previously been demonstrated in non-phagocytic cells (Touyz, Yao, & Schiffrin, 2003), however, the exact role of \( p40^{\text{phox}} \) remains in question. Lopes et al. (2004) and Sathyamoorthy et al. (1997) have reported \( p40^{\text{phox}} \) to be an inhibitor of NAD(P)H oxidase in neutrophils, (Lopes et al., 2004; Sathyamoorthy, de, I, Adams, & Leto, 1997) where as Kuribayashi et al. (2002) has reported it to be an activator, also in neutrophils (Kuribayashi et al., 2002). Lopes et al. (2004) were also able to demonstrate that this subunit is not necessary for activation.
Unlike p40\textsubscript{phox}, it has been well established through p47\textsubscript{phox-/-} mice (Lavigne, Malech, Holland, & Leto, 2001; Li & Shah, 2002a; Lavigne et al., 2001; Li et al., 2002a), as well as through inhibition of cytoskeleton assembly that p47\textsubscript{phox} is necessary for activation and has a direct interaction with actin in VSMCs. This interaction has been demonstrated to be through the actin-binding protein cortactin as disruption with cytoskeleton inhibitors was able to inhibit ROS generation due to Ang III (Touyz, Yao, & Schiffrin, 2005; Touyz, Yao, Quinn, Pagano, & Schiffrin, 2005). This characteristic does not seem to be shared by all cytosolic subunits as p67\textsubscript{phox} and Rac2 were able to translocate effectively to the membrane bound cytochrome b558 despite cytoskeletal disruption in phagocytic cells (van, Anthony, Fernandez-Borja, & Roos, 2004).

The membrane bound subunits of NAD(P)H oxidase, gp91\textsubscript{phox} and p22\textsubscript{phox} do not require any translocation for activation, but the gp91\textsubscript{phox} subunit does have an unique characteristic in that it is unstable in the absence of p22\textsubscript{phox}, and in patients deficient in p22\textsubscript{phox}, no phagocytic gp91\textsubscript{phox} can be detected in isolated neutrophils (Parkos, Dinauer, Jesaitis, Orkin, & Curnutte, 1989), suggesting a constitutively association (Bedard & Krause, 2007).

### II.3. Gp91\textsubscript{phox} homologues

Homologues of the original subunits for gp91\textsubscript{phox}, p47\textsubscript{phox} and p67\textsubscript{phox} of NAD(P)H oxidase have been identified. However, research has recognized that the gp91\textsubscript{phox} subunit is the chief subunit that influences the remaining components. The gp91\textsubscript{phox} subunit contains: NAD(P)H and molecular oxygen binding sites as well as flavin and heme groups which allow for electron transport between the two compounds (Babior, 1999). Aside from the phagocytic gp91\textsubscript{phox} homologue, NOX2 (NAD(P)H OXidase),
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which was the first identified homologue in phagocytes (Volpp, Nauseef, & Clark, 1988), there exists four other homologues, NOX1, 3, 4 and 5, three of which have been isolated in the vessel wall (Touyz et al., 2005). The first homologue identified in the vasculature, specifically VSMC, was NOX1 (Lassegue et al., 2001), originally known as MOX1 (Mitogenic OXidase). The other three homologues, NOX3, 4 and 5 were initially discovered in various tissues, human colon epithelial cells (Kikuchi, Hikage, Miyashita, & Fukumoto, 2000), human kidney cells (Geiszt, Kopp, Varnai, & Leto, 2000) and fetal tissue/adult spleen/uterus (Cheng, Cao, Xu, van Meir, & Lambeth, 2001) respectively. Combined research, primarily from the cardiovascular and immune systems, has given considerable insight into the similarities and differences in function between all NOX homologues. There are truly four key conserved structural properties within the NOX family. These include: a NAD(P)H binding region at the COOH terminus, a flavin adenine dinucleotide (FAD) binding region close to the COOH terminus transmembrane domain, 6 conserved transmembrane domains, 4 conserved heme-binding histidines, 2 of which are located in the 3rd and 2 in the 5th transmembrane domains (Bedard et al., 2007). Apart from these similarities, there are several differences among the NOX family.

II. 3.A. NOX1

NOX1 and NOX2 are the closest related in the homologue family with there being a ≈60% protein sequence identity (Banfi et al., 2000) and both requiring the p22phox subunit for functioning (Ambasta et al., 2004). NOX1 is distinct in that it can associate with slightly different cytosolic subunits, these being NOXO1 (NOX Organizer), p47s homologue (p47phox is sometimes referred to as NOXO2) and NOXA1 (NOX Activator), p67s homologue (Bedard et al., 2007). Having stated this, a study using transfected cells
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demonstrated the neither p47\textsuperscript{phox} nor p67\textsuperscript{phox} are specific for NOX2, and that ROS production can occur with only NOX1, p47\textsuperscript{phox} and p67\textsuperscript{phox} present (Banfi, Clark, Steger, & Krause, 2003).

II.3.B. NOX3

The research regarding NOX3 still remains in it infancy with its role in vivo still in question (Bedard et al., 2007). The amino acid sequence of NOX3 is quite similar to NOX1 and 2 with \( \approx 56\% \) conservation compared with NOX2, and hydrophobic plot analysis have suggested a very similar structure to the aforementioned homologues as well (Cheng et al., 2001). NOX3 is another p22\textsuperscript{phox} dependent enzyme and also associates with NOXO1, with p47\textsuperscript{phox} and p67\textsuperscript{phox} also having the capability to allow activation (Ueno, Takeya, Miyano, Kikuchi, & Sumimoto, 2005) similar to NOX1. Data still remains inconclusive as to the role of the GTPase Rac as a regulating subunit and the exact role of NOXA1, as Ueno et al. (2005) concluded that there was no role while Ueyama, Geiszt and Leto (2006) have presented data demonstrating the need for the subunit for proper functioning (Ueyama, Geiszt, & Leto, 2006).

II.3.C. NOX4

Originally named RENOX (REnal NOX) due to its high level of expression within the kidney epithelium (Geiszt et al., 2000), NOX4 appears to be a much more distant relative of NOX2, sharing only \( \approx 39\% \) protein homology (Bedard et al., 2007). As with the NOX1-3, NOX4 is p22\textsuperscript{phox} dependent (Ambasta et al., 2004), however, through use of transfection with several cell types, it has been shown that NOX4 does not require association with any cytosolic subunits for production of ROS (Martyn, Frederick, von, Dinauer, & Knaus, 2006). Unfortunately, data in regards to the GTPase Rac is not as
conclusive, with separate studies demonstrating contracting results (Martyn et al., 2006; Gorin et al., 2003).

II.3.D. NOX5

The final NOX in the gp91phox family has a unique set of characteristics. This homolog does not require any cytosolic or membrane bound subunits to become active, but activation is mediated by increases in intracellular Ca\(^{2+}\). The binding of Ca\(^{2+}\) to the binding domain is thought to lead to a conformation change allowing an interaction to occur between the COOH terminus and the binding area (Banfi et al., 2001).

II.4. Vasculature versus phagocyte NAD(P)H oxidase

As discussed earlier, the ability of ROS to participate in physiological and pathophysiological signaling makes it an appealing issue to researchers and as the prominent ROS producer in the vessel wall, NAD(P)H oxidase has received considerable attention (Touyz et al., 2004a). NAD(P)H oxidase has been shown to be active in all three regions of the vasculature to varying degrees due to various cytokines and growth factors. This includes the endothelium (Muzaffar, Jeremy, Angelini, Stuart-Smith, & Shukla, 2003), the media (Berry et al., 2000), and the adventitia (Di et al., 1999; Pagano et al., 1995).

Through the research that has been conducted, it has also become evident that phagocytic and vascular (non-phagocytic) NAD(P)H oxidase have distinct characteristics. Two principal differences between the two forms lie in the method by which the enzyme assembles as well as where the superoxide is released. The primary objective of phagocytic NAD(P)H oxidase is to create a short, concentrated burst of ROS to help kill any pathogens that were engulfed by the cell. In phagocytic cells, assembly of the
subunits must be rapid, ROS production must be brief and activity must lead to a lethal amount of superoxide (Babior et al., 2002). The responsibility of NAD(P)H oxidase is significantly different in all vascular cells.

The main purpose of the ROS producing enzyme is to produce intracellular signaling molecules. To maintain constant production of ROS for signaling, the vascular NAD(P)H oxidase is preassembled and constitutively active. Despite the constant activity of NAD(P)H oxidase, the actual concentration of ROS produced, particularly superoxide, remains at a relatively low level compared to its phagocytic counterpart (Lassegue & Clempus, 2003; Li et al., 2002a).

Another principal variation between the phagocytic NAD(P)H oxidase and its vascular equivalent is the NOX homologues that are involved. Having explored the variations that do occur in the 5 NOX subunits, it is important to note that the phagocytic NAD(P)H oxidase solely utilizes the prototypical NOX2 subunit (Lassegue et al., 2003), while NOX homologues 1,2,4 and 5 have all been identified somewhere within the vessel (Griendling, 2004). Distinguishing the exact location in the vessel, as well as subcellular location of these homologues has proved difficult due to a lack of high quality antibodies that have undergone sufficient testing (Bedard et al., 2007).

II.5. NOX2 versus NOX4

II.5.A. NOX2

The first homolog documented in the vascular system was the prototypical NOX2 form of gp91phox, with immunohistochemical results demonstrating that the adventitia possesses a majority of NOX2 in the vessel, as well as the other membrane and cytosolic subunits required for proper functioning, p22phox, p47phox and p67phox (Pagano et al., 1997;
Wang et al., 1998). Subsequent research has established that NOX2 is not solely limited to adventitia, as significant mRNA and protein levels have been observed in endothelial cells (Furst et al., 2005; Higashi et al., 2003), as well as to some extent in VSMC, particularly in VSMC from resistance arteries vessels (Touyz et al., 2002). Data does suggest varying expression dependant on the location of VSMC isolation, as conduit vessels do appear to express primarily the NOX1 isoform and NOX2 only at low or insignificant levels (Lassegue et al., 2001). As stated previously, the NOX1 and 2 homologues share very similar genes and amino acid sequences, thus, it is not surprising that both isoforms are located in the plasma membrane, similar to the placement of NAD(P)H oxidase in phagocytic cells. Within the membrane, NOX1 and 2 are principally associated with membrane ruffles, lamellipodium (leading edges) and focal complexes (Hilenski, Clempus, Quinn, Lambeth, & Griendling, 2004; Ushio-Fukai, 2006; Van Buul, Fernandez-Borja, Anthony, & Hordijk, 2005). Additionally, according to predictions based on amino acid sequences, there is also the possibility that they may reside in the membrane of the endoplasmic reticulum (ER) (Bengtsson, Gulluyan, Dusting, & Drummond, 2003), however this has never been demonstrated.

II.5.B. NOX4

The remaining isoform that has been identified in the vasculature is the NOX4 homologue. This isoform has been shown to be present in both ECs (Ago et al., 2004), VSMCs (Hilenski et al., 2004) and adventitial fibroblasts (Guo et al., 2006) and is thought to have the highest level of expression of all NOX isoforms (Krause, 2004). The location of the NOX4 homologue has generated interest as predictions for cellular location using amino acid sequences have suggested a place in the ER membrane
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(Bengtsson et al., 2003). This prediction has been supported in ECs both through tranfection with green fluorescent protein (GFP) tagged NOX4 (Ambasta et al., 2004; Van Buul et al., 2005) or strictly when using visualizing NOX4 through immunofluorescence (Martyn et al., 2006). Hilenski et al. (2004) in VSMCs and Kuroda et al. (2005) in ECs provided evidence to contradict these findings and used several techniques to demonstrate that the NOX4 homologue may locate within the nucleus. These results are intriguing as it would be unusual for a protein that typically spans a membrane six times to reside in a location that is membrane-free space. One theory provided for the observed expression of NOX4 at the ER membrane in transfected cells is that this localization may be caused by a deposit developing at the source of production, thus not an accurate representation of a normal cell (Bedard et al., 2007).

Other captivating research regarding NOX4 also demonstrates the potential differences that exist between the NOX2 and NOX4 enzyme. Initial ROS readings with transfected NOX4 have suggested that this particular isoform primarily produces the ROS H$_2$O$_2$, not the expected superoxide (Martyn et al., 2006). An explanation for this phenomenon that has been proposed hypothesizes that the present ROS assay techniques are unable to quantify and identify the initial ROS produced due to NOX4s location within an organelle, be it the ER or the nucleus. Once the superoxide is produced in the lumen of the organelle, conversion occurs to H$_2$O$_2$ allowing the non-polar molecule to migrate past the membrane (Bedard et al., 2007). Couple this hypothesis with the absence of a direct superoxide assay test by Martyn et al. (2006) and these results, although provocative, should not be considered direct evidence of a solely H$_2$O$_2$ producing NAD(P)H oxidase. More recent research regarding the species of ROS
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produced both supported the observed increase by Martyn et al. (2006) but also gave
credence to the hypothesis presented by Bedard & Krause (2007). In the study by Martyn
et al. (2006), H$_2$O$_2$ was the predominant ROS released by the cells. When examined
further by Serrander et al. (2007) using 3 different superoxide assays, it is evident that
there is an increase in superoxide in intracellular compartments as a robust signal was
observed upon transduction of NOX by nitro blue tetrazolium (NBT), but not
dihydroethidium (DHE) or electron spin resonance (ESR) (Serrander et al., 2007).

Another aspect regarding NOX4 that has garnered attention is the mechanism by
which the enzyme is regulated. As previously stated in respect to NOX1 and 2, both
isoforms are dependent on various subunits for functioning of NAD(P)H oxidase, and
thus, the production and/or inhibition of these subunits, including p22$^{pox}$, p47$^{pox}$, p67$^{pox}$
(or their equivalents, NOX01 and NOX1A1 respectively), Rac1 and potentially p40$^{pox}$,
allows for regulation of the enzyme (Bedard et al., 2007; Touyz et al., 2004a). Due to the
lack of requirement for cytosolic subunits (Geiszt et al., 2000; Martyn et al., 2006), as
well as the inconclusive nature of reported results regarding GTPase Rac requirements
(Gorin et al., 2003; Martyn et al., 2006), there is some question as to the mechanisms
used to regulate NOX4. Similar to other NOX isoforms, this homologue is thought to be
a constitutively active and possibly responsible for a majority of baseline ROS (Cucoranu
et al., 2005). Further research with various agonistic compounds, such as Ang II, have
demonstrated the potential for increased ROS production due to NOX4 upon stimulation
(Gorin et al., 2003). More recent investigation via Serrander et al. (2007) using
transduction of NOX4 has demonstrated that cytosolic subunits are not required, and
ROS production is dependent on the level of mRNA expression. The issue of regulation
is one that will surely be continued to be studied due the possible therapeutic benefits of conceivably inhibiting one solitary isoform.

It is also apparent that the different subcellular locations of the enzymes influence each individual function. Of particular interest is the potential for NOX4 to be a crucial enzyme in the regulation of oxidative stress-responsive gene expression, due to its subcellular location in either the perinuclear region in the ER (Ambasta et al., 2004; Martyn et al., 2006; Van Buul et al., 2005) or directly in the nucleus (Hilenski et al., 2004; Kuroda et al., 2005). Redox-dependent genes have previously been linked to cell growth, differentiation, senescence and apoptosis (Ushio-Fukai, 2006).

In summary, the ability of NAD(P)H oxidase to produce ROS in the vasculature has been extensively studied and the new direction of this research lies in understanding the functions of each NOX isoform. Subcellular locations are one clue as to what functions an isoform may regulate.

II.6. NOX4 and function within the vessel

The concept of specific roles for individual NOX homologues is a recent hypothesis in the field of signal transduction and the results produced are varied. Previous research that had suggested that activation of NOX4 is of importance examined the role that the homologue may have in phenotypic differentiation in cardiac and adventitial fibroblasts and VSMCs. Having previous results demonstrating that the highest mRNA levels for an NOX homologue in fibroblasts is NOX4 (Sorescu et al., 2002), the function of NOX4 in the process of differentiation from fibroblast to myofibroblast was examined in human cardiac fibroblast culture. Through the use of small-inhibiting ribonucleic acid (siRNA) specific for NOX4, researchers were able to
inhibit the phenotypic differentiation between cardiac fibroblasts and myofibroblasts due to TGF-β. In this study, of all the NOX isoforms present in the vasculature, only NOX4 was upregulated following TGF-β stimulation (Cucoranu et al., 2005).

A similar study with rats has recently demonstrated the importance of NOX4 in maintaining the phenotypic differentiation of VSMCs in vitro, as once again depletion of NOX4 protein levels through siRNA or increased passage number lead to a decrease in levels of VSMC markers α-actin, MHC, heavy caldesmon and calponin (Clempus et al., 2007). Other investigation regarding NOX4 has suggested that the proliferation of SMC in the pulmonary artery due to TGF-β is mediated specifically through the NOX4 isoform, as much like the previous studies, siRNA coded for NOX4 inhibits this proliferation (Sturrock et al., 2006).

Apart from these in vitro results, in vivo research has also indicated at least the possibility of involvement of NOX4 in the development of neointimal formation following balloon injury in rat carotid arteries. The study, conducted by Szocs et al. (2002), revealed a two-fold increase in NOX4 expression 15 days following the balloon injury, with significant amounts of NOX4 present in the neointima.

Despite the considerable amount of research linking upregulation of NOX4 to various cellular functions, other research has varying conclusions. While examining the role of NAD(P)H oxidase isoforms in cultured rat VSMCs, Lassegue et al. (2001) found that Ang II, as well as PDGF treatment actually decreased the mRNA levels of NOX4 and upregulated NOX1. A subsequent study by Ellmark et al. (2005) has supported these mRNA results and has demonstrated that thrombin and interleukin-1β (IL-1β) also lead to downregulation of the NOX4 isoform in VSMCs (Ellmark, Dusting, Fui, Guzzo-
Pernell, & Drummond, 2005). With respect to adventitial fibroblasts specifically, the phenotypic conversion from adventitial fibroblast to myofibroblast is one that garnered interest, particularly due to the work completed in cardiac fibroblasts. Presently, the research that has been produced has displayed an increase in NOX4 protein 12hrs following TGF-β1 treatment, despite contradicting gene chip analysis and reverse transcriptase polymerase chain reaction (RT-PCR) results as to whether or not there was an increase or decrease in the NOX4 gene expression during the phenotypic conversion (Guo et al., 2006). Similar to these results, Haurani et al. (2008) demonstrated that over expression of NOX4 lead to a decrease of endogenous NOX4 mRNA, suggesting a negative feedback cycle. The results also showed that NOX4 is involved in the myofibroblast migration process primarily through a H₂O₂-dependent mechanism (Haurani, Cifuentes, Shepard, & Pagano, 2008).

Due to the hypothetical location of NOX4 in the nucleus, it is rational to believe that this specific isoform would have a key role in signaling more so than its NOX relatives situated elsewhere within the cell, although evidence to this point is somewhat vague as to the exact responses, it is apparent that the gp91phox homologue NOX4 has a significant role within the vasculature.

III. Vasoactive Peptides: Angiotensin II and Endothelin-1

The vascular system utilizes a vast array of mechanisms to organize the complex physiological events of regulating proper blood flow to the necessary tissues. As mentioned previously, one method is via sympathetic nerve innervation directly into the vessel, another mechanism employed is the use of the vasoactive hormones the can
function in an endocrine, paracrine or autocrine manner and dictate physiological or pathological responses. Of this group of vasoactive hormones, the review will examine two peptides that have been demonstrated to have physiological and pathological functions in the vessel environment, particularly in regards to their ability to cause an increase in ROS and the subsequent development of oxidative stress, Ang II and ET-1.

III.1. Angiotensin II

III.1.A. Overview of Angiotensin II

The role of Ang II and the renin-angiotensin system (RAS) in the cardiovascular system cannot be overstated. Ang II, the final peptide product of a complex enzymatic proteic cascade, is involved in regulation of numerous vital functions, including cell growth, migration, ECM deposition, vasoconstriction and vascular remodelling (Mehta & Griendling, 2007). With such a central role in the vascular system, it is of no surprise that imbalances within the RAS occur, pathological consequences can ensue. Proof of the importance of Ang II is evident in treatment of several cardiovascular diseases, including hypertension and atherosclerosis, as front-line therapy now includes the use of both angiotensin converting enzyme (ACE) inhibitors and AT1 receptor inhibitors, one of the two receptors which mediates Ang II’s effects. Through the use of these pharmacological interventions, physicians have been able to lower blood pressure (BP) (Ferrario, 2006), vascular hypertrophy or hyperplasia (Volpe et al., 2002), ROS levels (Ghiadoni et al., 2003) and a host of other symptoms related to imbalances in Ang II (Ferrario, 2006). In humans, studies have shown that high renin levels, the initial enzyme in the RAS, and thus high Ang II levels are predictive of development of hypertension (Watt et al., 1992; Harrap et al., 2000) and subsequent rat models with direct Ang II
infusion have produced hypertensive animals that are used to study various responses related to Ang II in the vasculature.

One such response that has become evident is the production of substantial amounts of ROS (Laursen et al., 1997; Virdis, Neves, Amiri, Touyz, & Schiffrin, 2004). A consequence of this ROS is the inactivation of endothelium-derived nitric oxide, a major vasodilator, by superoxide (Rey et al., 2002). Additionally, several species of ROS are involved in harming cell signaling pathways.

**III.1.B. Angiotensin II and Reactive Oxygen Species Cell Signaling**

As previously discussed, oxidative stress was solely thought to be problematic due to its ability to damage the structure of deoxyribonucleic acid (DNA), protein and lipids (Touyz et al., 2004a). The response of the vasculature to Ang II has given an example of how ROS not only are detrimental due to there reactivity they have with various components of the cell, but also due to the effect they have in the signal transduction pathways associated with physiological and pathophysiological functions.

Initial studies conducted in the area of Ang II-induced ROS generation in the vasculature were met with scepticism and research regarding the concept of ROS generation was not well supported. It has now been 13 years since the initial study by Pagano et al. (1995) documenting NAD(P)H oxidase in the adventitia and research regarding Ang II-induced ROS is now occurring at a tremendous pace. Below is a brief history of the developments and discoveries that have occurred in the past 13 years.

Following the preliminary study demonstrating the existence of a functional NAD(P)H oxidase in the adventitial layer (Pagano et al., 1995), research focused on the mechanisms of activation and potential extracellular functions of the ROS production. At
this point, it was determined that ROS production could be stimulated by Ang II treatment in cultured adventitial fibroblasts and that the increase could be attributed to a NAD(P)H oxidase (Pagano et al., 1997). In particular, it was observed that the cytosolic subunit p67phox was required for the rise in ROS due to Ang II stimulation (Pagano et al., 1997; Pagano, Chanock, Siwik, Colucci, & Clark, 1998) and that p67phox was upregulated, along with NOX2 in the whole aorta following Ang II stimulation (Cifuentes, Rey, Carretero, & Pagano, 2000). Following hypotheses drawn from the previous studies, the effect of ROS, specifically superoxide, was examined and concluded to be a paracrine mediator of vasoconstriction through inhibition of NO (Di et al., 1999; Wang et al., 1998).

Despite these in vitro and ex vivo results that clearly demonstrate the importance of the NOX2 homologue in ROS generation, work done in gp91phox knockout (KO) mice (NOX2 KO) confirm the complexity of the in vivo regulation of BP. In NOX2 KO mice, there was no significant decrease in BP following 6 days of Ang II infusion compared with a wild-type (WT) group, however, there was a decrease observed in the baseline BP of WT and KO mice. Other interesting data from the study of the NOX2 KOs was that although gp91phox KOs did not decrease BP, it inhibited medial hypertonphy in the Ang II-infused model (Wang et al., 2001). These findings were consistent with the data observed in carotid artery isolated from Ang II infused mice. In the study by Liu et al. (2004), vascular NOX2 was inhibited with siRNA specific for NOX2 in an Ang II infused model. Results from this study affirmed the lack of development of medial hypertrophy in the carotid artery (Liu, Ormsby, Oja-Tebbe, & Pagano, 2004). These results are somewhat distinct from the investigation that has been conducted with VSMC,
as well as fibroblasts in culture, as previously VSMC hypertrophy and fibroblast proliferation had been attributed to endogenous ROS, H$_2$O$_2$ and superoxide production respectively (Griendling, Minieri, Ollerenshaw, & Alexander, 1994; Irani et al., 1997; Zafari et al., 1998).

Recent research regarding this subject has used specific overexpression of catalase (Zhang, Griendling, Dikalova, Owens, & Taylor, 2005), NOX1 (Dikalova et al., 2005) and p22phox (Weber et al., 2005) in VSMCs and has produced intriguing results. In all accounts, increased levels of H$_2$O$_2$ in VSMCs lead to hypertrophy, however, overexpression of NOX1 was also able to increase BP (Dikalova et al., 2005). This is different from overexpression of p22phox or catalase as when both were overexpressed, they did not raise or lower BP respectively in response to Ang II (Weber & Brilla, 1991; Zhang et al., 2005). Through these results, it is clear that ROS signaling plays a role in the development of high blood pressure and subsequent hypertension, although the exact role of ROS from various vessel components remains in question.

In an effort to ascertain the functions of ROS in Ang II signaling, researchers have not only utilized molecular methods to manipulate NAD(P)H oxidase function, but have also examined the vasoprotective effects of endogenous antioxidants. One endogenous substance that is able to utilize it’s antioxidant mechanism to dilate vessels is adrenomedullin (AM), although, at present it is unclear how AM is able to accomplish this (Yoshimoto et al., 2005). AM is abundantly expressed by vascular ECs in the vasculature and cardiac fibroblasts in the heart and has been observed working in both an autocrine and paracrine manner (Minamino, Kikumoto, & Isumi, 2002; Tomoda et al., 2001). Treatment with exogenous AM has proved successful in inhibiting Ang II induced
ROS dependent inflammatory responses associated with NAD(P)H oxidase (Tomoda et al., 2001; Yoshimoto et al., 2005). One additional response that has been investigated is the fibrotic effects of Ang II treatment in the heart. Through AM treatment, the fibrotic effect of Ang II was repressed in the coronary artery with a majority of the decreases occurring in the adventitial region (Tsuruda et al., 2005). These results with an endogenous antioxidant demonstrate the significance of ROS signaling, but also the importance of vasoprotective compounds in hindering ROS’s effects.

The ability of antioxidants to prevent the inflammatory responses of Ang II is reliant on the inhibition of redox-sensitive inflammatory genes. The redox-sensitive inflammatory genes that have been identified in VSMC include: intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), monocyte chemoattractant protein-1 (MCP-1), osteopontin, tissue factor and PAI-1 (Griendling, Sorescu, Lassegue, & Ushio-Fukai, 2000). The research completed suggests that through the inactivation of phosphatases and activation of tyrosine kinases, NAD(P)H oxidase derived ROS is able to activate p38 MAK kinase (Ushio-Fukai, Alexander, Akers, & Griendling, 1998) and Akt (Gorin et al., 2003). This activation leads to the activation of transcription factors such as nuclear factor κB (NF-κB), activator protein-1 (AP-1), HIF-1α and signal transducers and activators of transcription (STAT) and subsequent induction of the aforementioned inflammatory genes (Brandes, 2003). An additional instance, where induction of genes was measured through mRNA production, was when antioxidants were used to inhibit redox-sensitive gene was in the production and release of one of the cascade compounds of Ang II that mediates several of Ang II observed effects, ET-1, within adventitial fibroblasts. Having previously demonstrated the
production of ET-1 by adventitial fibroblasts due to Ang II (An et al., 2006), both pharmacological antioxidants and overexpression of Copper/Zinc (Cu/Zn) SOD, an endogenous antioxidant, was capable of decreasing the messenger ribonucleic acid (mRNA) levels and release of ET-1 in adventitial fibroblasts culture (An et al., 2007), and comparable research in ECs has demonstrated the same results with herbal antioxidants (Lee et al., 2005). Similar to ET-1, TGF-β is known to mediate several effects of Ang II and TGF-β’s release can be inhibited by antioxidants as well (Tsuruda et al., 2005). Through the previously conducted research, it is apparent that ROS have a central role in the signal transduction pathways in not only adventitial fibroblasts, but throughout the cardiovascular system. These realities lead to the possibility of different NOX homologues having varying levels of ability in activating signal transduction pathways or redox-sensitive genes dependent on their cellular location (Brandes, 2003). This hypothesis leads to significant interest regarding the NOX homologue that potentially resides directly within the nucleus, NOX4, and its proximity to redox-sensitive genes (Hilenski et al., 2004; Kuroda et al., 2005).

III.2. Endothelin-1

III.2.A. Overview of Endothelin-1

The mechanism of response of the vasculature to Ang II partially relies on direct interaction of Ang II with the receptor, however a considerable amount is dependent on a cascade of compounds which Ang II triggers to be released or produced, such as ET-1. The range of actions in the vasculature of ET-1 is just shy of Ang II as a multitude of vascular responses can be traced back to ET-1. Presently known actions of ET-1 include:
vasoconstriction, increased blood pressure and vascular tone, which occur primarily due to ET-1-induced production of cytokines and growth factors, production of ECM proteins and amplification of the effects of TGF-β1 and PDGF (Luscher & Barton, 2000b). The range of effects is large, however, the real interest in ET-1 began when it was demonstrated that endothelins in blood levels were elevated in several cardiovascular diseases (Stewart, Cernacek, Costello, & Rouleau, 1992; Vierhapper, Gasic, Nowotny, & Waldhausl, 1990), and could predict survival rate in certain cardiovascular diseases (Omland, Lie, Aakvaag, Aarsland, & Dickstein, 1994).

A brief history and overview of production of ET-1 is a follows: In 1986, the group of Gillespie et al. reported the ability of endothelial cells to not only produce a vasodilator, but also a vasoconstricting substance, which they named endothelin (Luscher & Barton, 2000a). After this study in 1986, it took the group of Yanagisawa et al. two years to isolate this 21 amino acid peptide (Yanagisawa et al., 1988). In actuality, there are three such endothelin peptides: endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin (ET-3), all with different distributions in various tissues, however, research has pointed to ET-1 being the most predominant and potent of the three in the vasculature (Inoue et al., 1989b).

While all three ETs are encoded by different genes, all achieve active state through the same process (Inoue et al., 1989b). PreproETs are first transcribed, then translated, creating a 203 amino acid prepropeptide. This prepropeptide is then cleaved to the inactive 38 amino acid precursor big ET-1 which is finally cleaved by various isoforms of endothelin converting enzymes (ECEs) to its active isoforms. Although big ET-1 is produced in numerous cell types, the ECEs required to convert big ET-1 are
localized to endothelial cells (Xu et al., 1994), VSMCs (Maguire, Johnson, Mockridge, & Davenport, 1997), cardiomyocytes (Kobayashi et al., 1998) and macrophages (Minamino et al., 1997) in the vasculature. Another enzyme responsible for the conversion of ET-1 to both an active and inactive form (ET-1 21 amino acids versus ET-1 31 amino acids), albeit at lesser degree, is a chymase, first isolated from mast cells of the lung (Wypij et al., 1992). This chymase is not only interesting due to its ability to cleave ET-1 into two pharmacologically distinct ET-1s, but also as it has recently been discovered within vascular smooth muscle cells (VSMC), although its functions are not completely understood (Luscher et al., 2000a).

Being peptide hormones, ETs interact with specific receptors located on target tissues. As of this moment, there have been two receptors identified in mammalian cells, the ET_A and endothelin-type B (ET_B) receptors. Both the ET_A receptor and ET_B receptor are members of the seven transmembrane segment G-protein-coupled receptor superfamily (Arai, Hori, Aramori, Ohkubo, & Nakanishi, 1990), and share 63% amino acid homology (Sakurai, Yanagisawa, & Masaki, 1992).

Despite this knowledge regarding ET-1, the assortment of functions associated with the peptide have made it difficult for researchers to deduce which characteristics of ET-1 are hazardous (Luscher & Barton, 2000c). Investigation continues into this significant vasoactive peptide as it is well established that although ECs (Inoue et al., 1989b; Inoue et al., 1989a) are the primary producers of the peptide, the capability of VSMCs (Mohacsi, Magyar, Tamas, & Nanasi, 2004) and adventitial fibroblasts (An et al., 2006) to synthesize ET-1 has also been shown, demonstrating the opportunity for an interesting ET-1 system within the vessel.
III.2.B. Endothelin-1 and Reactive Oxygen Species

The role of all members of ROS in the functions of ET-1 within the vasculature still remain unclear, although research has demonstrated an increase in ROS in explanted aorta, as well as VSMC and EC stimulated with ET-1: VSMC (Wedgwood, Dettman, & Black, 2001), EC (Duerrschmidt, Wippich, Goettsch, Broemme, & Morawietz, 2000), and aorta (Loomis, Sullivan, Osmond, Pollock, & Pollock, 2005). One initial topic that has been investigated within the literature is the actual source of ROS due to ET-1 stimulation, and results have been less than conclusive. Preliminary studies demonstrated the involvement of the NAD(P)H oxidase enzyme as NAD(P)H oxidase inhibitors were able to significantly decrease the levels of superoxide produced (Li et al., 2003b; Loomis et al., 2005; Li et al., 2003a). Interesting research regarding the interaction of Ang II, ET-1 and NAD(P)H oxidase also substantiated the previous findings as in Ang II induced hypertension model and the DOCA-salt model of hypertension, an ET<sub>A</sub> receptor blocker was able to significantly reduced the levels of superoxide produced in the vasculature (Callera et al., 2003; Laplante, Wu, Moreau, & de, 2005; Li et al., 2003a). In the Ang II induced hypertensive model, the source of the superoxide was specifically linked to a NAD(P)H oxidase isoform which relies upon cytosolic subunits as the flavoprotein inhibitor apocynin was able to inhibit the last phase and prolonged increase in ROS associated with this animal model. These results suggest ET-1, through NAD(P)H oxidase, is the stimulus increasing ROS in this model (Laplante et al., 2005).

Contrary to these results, some studies have suggested that ROS production due to ET-1 can occur through the either xanthine oxidase, NOS uncoupling or through increased mitochondrial respiration. Initial studies using VSMC culture were able to
significantly reduce ET-1 derived ROS related activity through use of various mitochondrial inhibitors (Touyz, Yao, Viel, Amiri, & Schiffrin, 2004b). In an *ex vivo* study, Loomis et al. (2005) were able to block ET-1-induced effects by NAD(P)H oxidase inhibitors, as well as directly inhibit superoxide via treatment with tetrahydrobiopterin (BH4), the cofactor required for nitric oxide synthase (NOS) coupling, suggesting both NAD(P)H oxidase and NOS uncoupling in this setting. Due to the difficulty in trying to inhibit mitochondrial derived ROS without sacrificing the animal, Callera et al. (2006) strictly examined the levels of p47phox, p22phox, xanthine oxidase and endothelial NOS (eNOS)/COX-2 in the DOCA-salt model of hypertension but did not observe any increase related to ROS production. The increase that was observed was related to antioxidant defence mechanisms in the mitochondria, manganese SOD (MnSOD) (Callera, Tostes, Yogi, Montezano, & Touyz, 2006). More research by Viel et al. (2008) has implicated NAD(P)H oxidase, xanthine oxidase and mitochondrial ROS production due to ET-1 stimulation in DOCA-salt rats. In the *in vivo* study, NAD(P)H oxidase inhibitors, xanthine oxidase inhibitors, ET<sub>A</sub> receptor antagonists and combination ET<sub>A</sub> receptor and ET<sub>B</sub> receptor antagonists to a lesser extent, were able to decrease BP in DOCA-salt rats. The xanthine oxidase inhibitor and mitochondrial inhibitors were also able to inhibit ROS production when examined with fluorescence, suggesting a significant role for these enzymes in the production of ROS due to ET-1 stimulation (Viel, Benkirane, Javeshghani, Touyz, & Schiffrin, 2008). Notwithstanding the research that has been complete, the area regarding ET-1 and ROS production is still remains vastly uncharted.
Mechanisms of Endothelin-1

IV. Rationale

IV.1. Adventitia

The role of the adventitial layer of the vessel in pathological processes has been demonstrated by Ang II-induced hypertension (Wang et al., 2001), inactivation of NO (Wang et al., 1998) and neointimal formation (Dourron et al., 2005; Weaver et al., 2006). Recent research in hypoxia-induced pulmonary vascular remodelling supports the hypothesis that vessels are regulated through an ‘outside in’ mechanism with the adventitia initiating responses (Stenmark et al., 2006; Stenmark, Fagan, & Frid, 2006; Maiellaro et al., 2007). The above research demonstrates that the adventitia is more than just an innocent bystander in the progression of vascular disease.

IV.2. Endothelin-1

ET-1 is an important vasoactive peptide. ET-1 effects on the vasculature are regulated by ET$_A$ and ET$_B$ receptors (Luscher et al., 2000a). Although ET-1 is mainly produced by ECs, the production of ET-1 had been demonstrated in several other cell types. In addition, ET-1 has been reported to contribute to the vascular actions of Ang II both in vitro (Chen, McNeil, Wilson, & Gopalakrishnan, 1995) and in vivo (Rajagopalan et al., 1997).

My laboratory recently demonstrated the production of ET-1 in adventitial fibroblasts in vitro (An et al., 2006). We also demonstrated that this Ang II-induced ET-1 production is mediated through NAD(P)H-oxidase derived superoxide (An et al., 2007). Additionally, these studies demonstrated that Ang II-induced ET-1 has an autocrine action and leads to increased collagen deposition through the ET$_A$ receptor activation (An et al., 2007). These results illustrate the importance of adventitial ET-1.
IV.3. Reactive Oxygen Species

ROS are important secondary messengers mediating normal biological functions but can become detrimental to cells if over produced. All vascular cells have the potential to generate ROS. Recently, our laboratory and others have shown that the adventitia is the dominant source of superoxide generation in the aortas of mice (Wang et al., 2001; Wang, Johns, Xu, & Cohen, 2002), rats (Di et al., 1999; Wang et al., 1998), and rabbits (Pagano et al., 1995). Indeed, Ang II (Griendling et al., 2000; Touyz et al., 2004a) and ET-1 (Duerrschmidt et al., 2000; Fortuno et al., 2004) are known to increase vascular ROS generation leading to many changes in the cardiovascular system. However, whether or not ET-1 induces ROS production in adventitial fibroblasts has not been determined prior to studies in this thesis.

Extracellular-response kinase (ERK) is one of three major members of the mitogen-activated protein kinase (MAPK) family and is an important step in signal transduction of several growth factors, mitogens, neurotransmitters and hormones. The ERK1/2-MAPK signaling pathway is activated by Ang II and ET-1. Research conducted regarding the signaling of superoxide production has reported that activation of the ERK1/2-MAPK pathway is responsible for a majority of Ang II-induced superoxide produced in the vasculature (Ding, Chapman, Boyd, & Wang, 2007; Eguchi, Matsumoto, Motley, Utsunomiya, & Inagami, 1996; Lo, Shih, & Jiang, 2005). Unlike Ang II, ET-1-induced ROS response has been reported to be independent of the ERK1/2-pathway in VSMCs (Daou & Srivastava, 2004; Laplante & de, 2006), explanted aorta (aorta removed from body and cleaned of all connective and adipose tissue) (Laplante et al., 2006) or
cardiac fibroblasts (Cheng et al., 2003). In this thesis, I will test it for the first time if ERK1/2 pathway mediates ET-1-induced ROS in adventitial fibroblasts.

NAD(P)H oxidase is the major enzyme responsible for superoxide production by Ang II in the vasculature. Although gp91phox is substituted by its homologue mitogenic oxidase-1 in rat VSMC, rat adventitial fibroblasts and ECs and human VSMCs express gp91phox (NOX2). Using KO mice my laboratory has also shown that it is NOX2 that is chiefly responsible for Ang II-stimulated vascular oxidative stress and smooth muscle growth in vivo (Wang et al., 2001).

NOX4 is a more recently discovered gp91phox subunit homolog in the vasculature. Cucoranu et al. (2005) demonstrated the importance of NOX4 in the vasculature as specific inhibition of this enzyme was able to inhibit the phenotypic conversion from a cardiac fibroblast to a myofibroblast due to TGF-β stimulation. These results underscore the potential importance of NOX4 in vascular ROS production under pathological conditions. Although information regarding NOX2 has illustrated the importance of NAD(P)H oxidase-derived ROS, it is possible that NOX2 may not participate in all NAD(P)H oxidase-dependent signals. This suggests that there may be distinct signaling pathways mediated by distinct NOX isoforms. In this thesis, I will test the essential roles of these two major isoforms, NOX2 and NOX4, in ET-1-induced ROS in adventitial fibroblasts.

IV.4. Endothelin-1 and Reactive Oxygen Species

From prior research regarding other humoral factors such as Ang II, it is clear that one mechanism through which the signaling is mediated through the vessel is by the production of ROS. With regard to ET-1-induced ROS production, the literature is
limited to ECs (Duerrschmidt et al., 2000), VSMCs (Wedgwood et al., 2001; Laplante et al., 2005; Laplante et al., 2006), cardiac fibroblasts (Cheng et al., 2003) and explanted aorta (Loomis et al., 2005). Research has illustrated the significant role of Ang II-induced adventitial superoxide plays in vascular remodeling in vivo (Wang et al., 2001) and examining ET-1-induced ROS production in adventitial fibroblasts will supplement the information related to adventitial produced ROS. To commence this area of research, it is important to study the basic functions of ET receptors, signaling pathways and potential sources of ROS in ET-1-induced ROS as this information may aid in the development of treatments to help limit the pathological consequences of ET-1.
In light of the above information, I sought to determine what the ROS response to ET-1 would be in adventitial fibroblasts and investigated what pathway and NAD(P)H oxidase isoforms are involved in the ET-1 response. I also wanted to determine if there is an Ang II induced ROS response in adventitial fibroblasts and what receptors are involved in mediating this response. A research plan with 5 objectives was designed to examine these questions: (1) To examine whether ET-1 administered exogenously increases ROS levels. (2) To determine whether NOX2 contributes to the ET-1-induced increase in ROS levels. (3) To determine whether NOX4 contributes to an ET-1-induced increase in ROS levels. (4) To examine whether the ERK1/2-MAPK signaling pathway plays a role in the ET-1-induced increase in ROS levels. (5) To determine the role of the AT and ET receptors in the Ang II induced increase of ROS levels. A brief illustration of my hypotheses for the mechanism of ET-1 action is displayed in Figure 1 and a brief illustration of my hypothesis for the mechanism of Ang II action is displayed in Figure 2.
Mechanisms of Endothelin-1

Figure 1: Hypothesized mechanism of ET-1 action on aortic adventitial fibroblasts: ET-1 will increase the activity of ERK 1/2 MAPK signaling pathway via ET<sub>A</sub> receptors, activating and/or increasing the expression of NOX2 or NOX4 isoforms of NAD(P)H oxidase leading to an increase in ROS levels in adventitial fibroblasts.
Figure 2: Hypothesized mechanism of Ang II action on aortic adventitial fibroblasts:

Ang II-induced ROS production will occur in two phases following treatment with Ang II. These phases will be mediated by AT₁ and ETₐ receptors, respectively.
VI. Research plan

VI.1. Objective: To examine whether ET-1 administered exogenously increases ROS levels

I will test superoxide production in response to ET-1 by treating adventitial fibroblasts with ET-1. Explanted aorta (Loomis et al., 2005), VSMCs (Laplante et al., 2005; Wedgwood et al., 2001; Laplante et al., 2006), cardiac fibroblasts (Cheng et al., 2003) and cultured ECs (Duerrschmidt et al., 2000) have shown an increase in superoxide production with ET-1 stimulation. The role of ERK1/2-signaling pathway (Objective 4) and gp91phox homologues, NOX2 (Objective 2) and NOX4 (Objective 3) will also be examined.

VI.1.A. Objective: To determine the effect of ET-1 stimulation on superoxide levels, measured by lucigenin enhanced chemiluminescence

Cultured adventitial fibroblasts will be treated with ET-1 (10 nM) for varying lengths of time, from 0 to 360 min. Superoxide will be measured by lucigenin chemiluminescence. I predict that ET-1 will cause an increase in superoxide production in a time-dependent manner, peaking at 4 hrs. The treatment time with the most significant increase will give an optimal treatment time for the remaining objectives examining ET-1 stimulation and superoxide or ROS production.

VI.1.B. Objective: To determine the effect of ET-1 stimulation on ROS levels, measured by 2',7'-dichlorofluorescein fluorescence

Cultured adventitial fibroblasts will be treated with ET-1 (10 nM) for 4 hours (hrs). ROS will be measured by 2',7'-dichlorofluorescein (DCF) fluorescence. I predict
that the DCF results will verify lucigenin enhanced chemiluminescence results that ET-1 stimulates an increase in ROS production at 4 hrs.

**VI.1.C. Objective: To determine the role of ET$_A$ and ET$_B$ receptors in mediating ROS levels**

Cultured adventitial fibroblasts will be treated with ET-1 (10 nM) for the optimal treatment time attained in Objective 1 (4 hrs) in the presence or absence of ET receptor antagonists (BQ123, 1µM, ET$_A$; and BQ788, 1µM /100 nM, ET$_B$), respectively. Superoxide will be measured by lucigenin chemilumiscence and ROS by DCF fluorescence and results will be compared. I predict that ET-1-induced superoxide production will be inhibited by the ET$_A$ antagonist, but not the ET$_B$ receptor antagonist. These results will indicate that the ROS response is primarily mediated by the ET$_A$ receptor.

**VI.2. Objective: To determine whether NOX2 contributes to an ET-1-induced increase in ROS levels**

This objective will be tested in two ways: The effect of ET-1 on activation and expression of NAD(P)H oxidases in adventitial fibroblasts is unknown. Pharmacological inhibitors will be used to inhibit the production of ROS by NAD(P)H oxidases and the results of different inhibitors will be compared. Secondly, adventitial fibroblasts lacking the NOX2 isoform of NAD(P)H oxidase will be stimulated with ET-1 to determine if NOX2 plays a significant role in ET-1-induced ROS production.
VI.2.A. Objective: To determine the importance of NAD(P)H oxidase using pharmacological inhibitors

Cultured adventitial fibroblasts will be treated with ET-1 (10 nM) for 4 hrs. ROS production will be measured in the presence and absence of NAD(P)H oxidase inhibitors, diphenyleneiodonium (DPI) (10 μM) and apocynin (10 μM). ROS production will be measured by DCF fluorescence and results will be compared. Differences exist in the mechanism of inhibition between both DPI and apocynin. Apocynin, inhibits the assembly of the cytosolic components of the enzyme, including p47\textsuperscript{phox}, p67\textsuperscript{phox} and p40\textsuperscript{phox}. DPI is a much broader inhibitor its mechanism of inhibition allows no electron transfer on flavoprotein dependent sites, thus NAD(P)H oxidases that do not require cytosolic subunits are also inhibited. I predict that ET-1-induced ROS production will be significantly decreased by both inhibitors, suggesting that NAD(P)H oxidase participates in ROS generation.

VI.2.B. Objective: To determine the importance of NAD(P)H oxidase using NOX2 knockout mice

Cultured adventitial fibroblasts will be isolated from WT or NOX2 KO mice. The cells will be treated with ET-1 (10 nM) for 4 hrs. ROS production will be measured by DCF fluorescence and results from WT and KO mice will be compared. I predict that ET-1-induced ROS production will be significantly decreased in cells from NOX2 KO mice.
VI.3. Objective: To determine whether NOX4 contributes to an ET-1-induced increase in ROS levels

This objective will be tested via two methods (1) to examine NOX4 expression in response to ET-1 stimulation; and (2) using adventitial fibroblasts treated with siRNA for NOX4 and observe the ROS response to ET-1 stimulation.

VI.3.A. Objective: To determine NOX4 expression in response to ET-1 stimulation

Since the knowledge of NOX4 is very limited, I first will determine if NOX4 protein is present in mouse aortic adventitial fibroblasts and if the expression level increases in response to ET-1 treatment (10 nM). Cultured adventitial fibroblasts will be treated with ET-1 for varying lengths of time, from 0 to 24 hrs. NOX4 protein expression will be measured by Western blot. I predict that NOX4 expression will be upregulated due to ET-1 stimulation in a time-dependent manner.

VI.3.B. Objective: To determine the effect of NOX4 on an ET-1-induced increase in ROS levels

Adventitial fibroblasts will be treated with ET-1 (10 nM) for the optimal treatment time attained in Objective 1 (4 hrs). Cells will also be treated with or without siRNA specific for NOX4. Cellular superoxide production will be measured by lucigenin enhanced chemiluminescence. I predict that siRNA specific for NOX4 will decrease ET-1-induced ROS production, suggesting an important role of NOX4 in ROS generation.
VI.4. Objective: To examine whether the ERK1/2-MAPK signaling pathway is essential for ET-1-induced ROS production

In order to test this objective, I first will determine if ERK1/2 is activated by stimulation with ET-1 in adventitial fibroblasts. Activation of ERK1/2 via ET-1 stimulation has been shown in both VSMCs (Daou et al., 2004; Laplante et al., 2006; Touyz et al., 2004b) and cardiac fibroblasts (Cheng et al., 2003). If activation of the ERK1/2 pathway occurs, I will then examine if it is possible to inhibit ET-1-induced ROS production in adventitial fibroblasts by inhibiting the ERK1/2 pathway.

VI.4.A. Objective: To examine the ratio of phospho-versus total ERK1/2 in response to ET-1 stimulation

In the first experiment, I will test to determine if ERK 1/2 becomes phosphorylated in response to ET-1 in adventitial fibroblasts and at what time point this occurs. Cultured adventitial fibroblasts will be treated with ET-1 (10 nM) from 0 to 240 mins. Protein will be collected. The levels of phospho and total ERK1/2 will be measured by Western blot. The ratio of phospho- versus total ERK1/2 will be used as an indicator for ERK1/2 activity. I predict that ET-1 will increase phospho-ERK1/2 expression in a time-dependent manner.

VI.4.B. Objective: To examine whether the ERK1/2-MAPK mediates an ET-1-induced increase in superoxide levels, measured by lucigenin enhanced chemiluminescence

In the second experiment, I will determine if ET-1-induced superoxide production is mediated through the ERK1/2 pathway. Cultured adventitial fibroblasts will be treated with ET-1 (10 nM) for optimal treatment time attained in Objective 1 (4 hrs). Superoxide production will be measured in the presence and absence of MEK1/2 inhibitor PD98059.
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(10 μM) and its negative analogue SB202474 (10 μM). I predict that ET-1-induced superoxide production will be inhibited by the MEK1/2 inhibitor but not its negative analogue. These results will suggest that the ET-1-induced increase in ERK1/2 activity is partially responsible for mediating superoxide generation.

VI.5. Objective: To determine whether Ang II contributes to an increase in ROS levels and determine what role AT receptors and ET receptors have in mediating this increase.

It has been reported that adventitial fibroblasts have the ability to produce significant amounts of superoxide due to Ang II stimulation (Pagano et al., 1997; Wang et al., 1998) and that the Ang II-induced superoxide production leads to synthesis and release of functional ET-1, which contributes to increased collagen synthesis in adventitial fibroblasts (An et al., 2006). It has also recently been demonstrated that Ang II stimulation leads to ET-1-induced ROS production in VSMCs (Laplante et al., 2005). In this objective, I examined if Ang II increases superoxide and the role the AT and ET receptors play in mediating this production.

VI.5.A. Objective: To examine whether Ang II contributes to increased superoxide levels.

Cultured adventitial fibroblasts will be treated with Ang II (100 nM) for various time periods, from 0 to 360 mins. Superoxide will be measured by lucigenin enhanced chemiluminescence. I predict that ET-1 will cause an increase in superoxide production at or before 1.5 hr of Ang II treatment.
VI.5.B. Objective: To examine the role of AT₁ and AT₂ receptors in mediating an Ang II-induced increase in superoxide levels

Cultured adventitial fibroblasts will be treated with Ang II (100 nM) for 6 hrs. Superoxide will be measured by lucigenin enhanced chemiluminescence. Superoxide production will be measured in the absence or presence of Ang II receptor antagonists (Losartan, 100μM, AT₁; PD-123319, 100μM, AT₂). I predict that the AT₁ receptor will mediate the Ang II-induced increase in superoxide production.

VI.5.C. Objective: To determine the role of ETₐ and ETᵦ receptors in mediating an Ang II-induced increase in superoxide levels

Cultured adventitial fibroblasts will be treated with Ang II (100 nM) for 6 hrs. Superoxide will be measured by lucigenin enhanced chemiluminescence. Superoxide production will be measured in the absence or presence of ET-1 receptor antagonists (BQ123, 1μM, ETₐ; and BQ788, 1μM, ETᵦ). I predict that the ETₐ receptor will mediate the Ang II-induced increase in superoxide production.
VII. Materials

All materials and compounds, unless otherwise noted were purchased from Sigma-Aldrich (Oakville, Ontario). ET-1, BQ 123 (ET\textsubscript{A} receptor antagonist) and BQ 788 (ET\textsubscript{B} receptor antagonist) were purchased from American Peptide Company Inc. (Sunnyvale, California, USA). PD98059 (MEK1/2 inhibitor) and SB202474 (PD98059 negative analogue) were purchased from Calbiochem (San Diego, California, USA). Transfection reagents were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Anti-NOX4 antibody was generously donated by Dr. J.D. Lambeth of Emory University (Atlanta, Georgia).

Table 1: Antibodies Utilized for Western blotting

<table>
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<tr>
<th>Primary Antibody</th>
<th>Dilution in 5% Blocking buffer</th>
<th>Molecular Weight of Target protein (kD)</th>
<th>Secondary Antibody</th>
<th>Secondary Antibody dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Anti-Rabbit HRP conjugate</td>
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<td>Dr. J.D. Lambeth, Emory University</td>
</tr>
</tbody>
</table>
VIII. Methodology

VIII.1. Coating of cell culture dishes

Isolated primary adventitial fibroblasts were grown on 35 mm culture dishes which were pre-coated with a 0.1% poly-lysine solution. 0.3ml of the lysine solution was added to the culture dishes to completely cover the surface area of the dish. The dishes sat at room temperature for at least 30 mins. After this time period, the lysine solution was aspirated and left to dry.

VIII.2. Isolation and Primary cell culture of adventitial fibroblasts

Male C57B6 mice, 16 to 18 weeks of age, and male gp91phox KO mice were obtained from Jackson Laboratory (Bar Harbor, ME). The mice were anesthetized with isoflurane inhalation and then sacrificed by vertebral dislocation. Thoracic aortas were sterilely removed and cleaned. Connective tissue and perivascular fat was separated from the adventitia by dissection under a surgical microscope. The isolated adventitia was then cut into 1-2 mm² flat segments and planted on 0.1% polysine coated dishes. Fresh medium consisting of Dulbecco’s Modified Eagle’s medium and F-12 nutrient mixture (DMEM/F-12) with 15 mM Hepes and 2.5 mM L-glutamine (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 10 u/ ml penicillin, 10 µg/ml streptomycin and 25 ng/ml amphotericin B was added into each well (subsequently referred to as 10% FBS DMEM/F-12). The explants were incubated in a humidified incubator at 37°C in a 95% air/5% CO₂ atmosphere until the cells were confluent, typically 5-7 days. Confluent cells were subsequently harvested for passage with a trypsin (0.05%) and ethylenediamine tetraacetic acid (EDTA) (0.02%) solution. When cells from passage 1 or 2 had reached sub-confluence, they were frozen with a
90% FBS and 10% DMSO mixture and kept at -80°C overnight, then kept in -196°C until further use. All attempts were made to utilize adventitial fibroblasts that were not frozen. Cells which had previous been frozen were only used for Objective 1.A., 1.C., 4.B.

Subcultures of 3 passages were used in my experiments. The responses are consistent among the cells from passage 1 to passage 3 when the cell passage ratio was 1 to 3. Coating of dishes with 0.1% polysine was only completed for the initial culture dish. Subcultures did not required polysine treated dishes for successful growth. This method has previously been published and characterization of cells was completed to ensure the isolation and culturing of adventitial fibroblasts (An et al., 2006). Briefly, specific cell markers for ECs, VSMCs and leukocytes were absent when cultured cells were tested. Absence of these cell types, plus the presence of vimentin, a non-specific cell marker suggested that the cells cultured were adventitial fibroblasts. This topic is discussed further in XI.1.

All animal protocols were approved and conducted according to the recommendations from Research Sub-Committee of Brock University on Animal Care and Use and Canadian Council on Animal Care.

VIII.3. Method for cell passage

When cells were grown on tissue culture dishes and reached confluency (typically 10 days for P0-P1) and 4-6 days for subsequent passages, medium was aspirated and cells were washed 3 times in sterile phosphate buffered saline (PBS). Cells were then trypsinized with 0.05% trypsin/0.02% EDTA allowing for the surface to be covered for approximately 2-3 mins with gentle agitation. Culture dishes were checked under the microscope to ensure that the cells were detached. Once detached, 10% FBS DMEM/F-
12 was added into each well to inactivate the trypsin reaction. The now detached cells plus medium were transferred to a sterile 50mL conical tube and centrifuged at 2200 rcf at 4°C for 8 mins. The cell pellet was then resuspended in an appropriate amount of 10% FBS DMEM/F-12 and aliquated to a 75mm tissue culture flask or 35mm culture dish. Cells were then incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

VIII.4. Method for cell treatment

When cells were at least 85% confluent, cells were quiescented by reducing the serum concentration of the growth medium to 0.1% FBS DMEM/F-12 for 24 hrs. Quiescenting cultured cells induces syncronization of the cell cycles. Once quiescent, cells were washed 3 times in sterile PBS. Drugs were made to the required concentrations in 15ml conical tubes with the 0.1% FBS DMEM/F-12 medium. Before treatment of the cells occurred, medium and drug solutions were added to the cells, the drug and medium solution were filtered with 0.2 μm syringe filters to remove impurities. Prior to addition of medium to conical tubes, syringes and syringe filters, all were rinsed with 3 mg/ml bovine serum albumin (BSA). The medium supplemented with chemicals was then added to the cells, which were then placed back in the humidified atmosphere incubator.

VIII.5. Method for cell lysis

Once cells were stimulated with Ang II or ET-1 and their various antagonists and inhibitors, the supernatant was removed and the cells were washed 3 times in sterile PBS and placed on ice. 35 uL of 1% cell lysis buffer (Cell Signaling, Beverly, MA, USA), which consisted of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA (metal ion scavenger), 1 mM EGTA (scavenge Mg²⁺ and Ca²⁺), 1% Triton (detergent), 2.5 mM sodium pyrophosphate (protease inhibitor), 1 mM beta-glycerophosphate (phosphotase
inhibitor), 1 mM Na₃VO₄ (phosphatase inhibitor), 1 μg/ml leupeptin (protease inhibitor)
and supplemented with 1mM PMSF (serine protease inhibitor) was added to each culture
dish. Cells were incubated for 10 mins, dishes scraped and the remaining solution was
collected. The collected solution was then briefly sonicated, and then centrifuged for 20
mins at 10600 rcf at 4°C. Supernatants were then removed from the cell debris. Aliquots
were taken for protein concentration determination and the remaining solution stored in
the freezer set at -80°C.

VIII.6. Measurement of sample protein concentrations

Protein concentrations were determined with the use of the BIO-RAD Bradford
Reagent. The procedure is based on the formation of dye (Coomassie Blue G)-protein
complex, which can cause a shift in the absorption maximum from 465 to 595nm. The
amount of absorption is proportional to the protein present. The assay yields a linear
response from 1-140μg/ml based on the BSA as the protein standard. Serial dilutions
were made according to the following procedure.

BSA standard solution
1. BSA stock solution: 10mg/ml (40mg BSA/4ml distilled deionized water),
   aliquoted (100μl) frozen at -20°C.
2. BSA work solution: 1mg/ml (100μl stock + 900ul DDI water)
3. BSA standard solution: 0.5mg/ml (50μl work + 50μl water)
   0.4mg/ml (40μl work + 60μl water)
   0.3mg/ml (30μl work + 70μl water)
   0.2mg/ml (20μl work + 80μl water)
   0.1mg/ml (10μl work + 90μl water)
   0.05mg/ml (20ul 0.1mg/ml + 20ul water)

10μl of each standard was separated into separate microtiter plate wells. Protein standards
and samples were assayed in triplicate. 200μl of the Bradford Reagent diluted in a 1:4
water ratio was then added to each well and allowed to incubate at room temperature for
5 mins (no longer that 1 hr). Absorbance values were then determined at 595nm using
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KC4 software and a SynergyHT (Biotek, Vermont, USA) microplate reader. Absorbance values were then exported to an Excel spreadsheet and sample concentrations were determined from a standard curve created from the known standard protein concentrations.

VIII.7. Measurement of protein levels by Western blot

The Western blot protocol to be used in the proposed study has been published previously (An et al., 2006; An et al., 2007; Ding et al., 2007).

Following treatment of adventitial fibroblasts with Ang II, ET-1 and related inhibitors and antagonists in 35 mm dishes, cells were lysed with lysis buffer and the solution collected (protocol IX.5). Protein concentrations of the samples were calculated using the BIO-RAD Bradford Reagent (protocol IX.6). Equal amounts of total proteins (10-20 μg) were calculated. Equal total protein samples were then diluted with 5X sodium dodecyl sulphate (SDS) sample buffer and boiled for 5 mins. Samples were then electrophorized at 120V for 2 hrs (or until sample buffer dye reached gel bottom) in 7% SDS-polyacrylamide gel electrophoresis (PAGE) under conditions which eliminate secondary and tertiary protein structures. These conditions are also known as reducing conditions. The gels were then transferred to 0.2um polyvinylidene difluoride (PVDF) membranes at 300mA for 1.5 hrs (BIO-RAD). Membranes were then washed in PBS for 5 mins, then incubated in blocking buffer containing 5% non-fat dry milk and PBS- with 0.1% tween (PBS-T) for 1 hr. Membranes were then washed in PBS-T 3 times 5 min increments. Primary antibodies were then added at the appropriate dilutions. Antibodies and membranes were incubated overnight at 4°C. The membranes were then washed 3 times in 5 min increments in PBS-T and then incubated secondary peroxidase-conjugated
antibody diluted to a 1:2000 concentration and subsequently detected by the ECL detection system or Flurometer (Amersham Pharmacia Biotech) according to manufacturer’s instruction.

**VIII.8. Reactive oxygen species measurement**

**VIII.8.A. Lucigenin enhanced chemiluminescence**

For superoxide measurements, adventitial fibroblasts were cultured in 35 mm culture dishes (BD Falcon, Mississauga, Ontario). The Turner Biosystems luminometer TD 20/20 was adapted and used for chemiluminescence measurement by lucigenin. Lucigenin added to the cells gains an electron from superoxide and emits a photon of light. After the addition of the lucigenin, there is 10 min incubation at 37°C before readings should occur. The TD 20/20 reads and displays arbitrary units of light. Measurements were taken at 30 second intervals throughout a 5 min period. Five μM (final concentration) of lucigenin was added into cells. Tiron (10 mM, final concentration), a phenolic compound added was then added to quench all superoxide-dependent chemiluminescence. Tiron is able to scavenge superoxide due a hydroxyl group that losses a positive hydrogen ion when is reacts with superoxide. Five mins incubation with tiron at 37°C occurs to ensure that all superoxide is quenched. The superoxide production was expressed as milli units of light per unit of time and cell protein. Cell protein determination was determined with BIO-RAD reagent as explained in IX.6.

Despite the wealth of knowledge gained in the vascular system through the use of the lucigenin assay in the detection of superoxide levels, controversy stills remains due to
the possibility for redox-cycling (Dikalov, Griendling, & Harrison, 2007). Redox-cycling is the production of superoxide due to lucigenin: $\text{LC}^- + \text{O}_2 \rightarrow \cdot \text{O}_2^- + \text{LC}^{2+}$, however, the final concentration of lucigenin used in my assay (5µM) is below the levels demonstrated to cause redox-cycling and stimulation of ROS production in vascular tissue (>20µM) (Li et al., 1998; Skatchkov et al., 1999; Tarpey et al., 1999) and is reported as a valid method for superoxide detection in vascular tissue (Dikalov et al., 2007; Munzel, Afanas'ev, Kleschyov, & Harrison, 2002).

Lucigenin chemiluminescence was intended to be the lone measurement used in detection of superoxide levels. This was due to lucigenin’s specificity for superoxide and the quantatitive method through which results are attained. During the completion of the project, the readings obtained from the luminometer with 5 µM lucigenin became increasing sporadic and unreliable. Due to these variations in superoxide measurements with lucigenin, a less specific and more qualitative probe, 2',7'-dichlorofluorescein diacetate (DCF-DA), was utilized for the remaining experiments. Lucigenin results in Objective 1.A after 4 hrs of ET-1 stimulation were verified with DCF fluorescence (Objective 1.B) to ensure comparable results between the two methods. Objective 1.C utilized a combination of both DCF fluorescence and lucigenin due to a small number of lucigenin samples. It was also my hope that DCF fluorescence could verify Objective 4.B as only 2 samples were completed for treatment groups involving SB202474, the negative analogue of PD98059. Unfortunately, due to limited resources, Objective 4.B was not able to be verified with DCF fluorescence. For the remaining lucigenin experiments, trials that had a satisfactory sample size were not duplicated.
VIII. 8.B. 2',7'-Dichlorofluorescein diacetate

DCF-DA is one of the most common probes used to detect intracellular ROS. Once DCF-DA enters the cell, DCF-DA is cleaved to 2',7'-dichlorofluorescein (DCF) and subsequently reacts with a host of ROS. The most prominent molecule that DCF interacts with is H₂O₂. Despite DCF primarily reacting with H₂O₂, DCF induced fluorescence includes reactions with all ROS. DCF is considered a superoxide detection method as a majority of DCF’s primary target H₂O₂ is the secondary product of superoxide. Thus DCF fluorescence can be used to estimate intracellular superoxide levels (Dikalov et al., 2007). As DCF is primarily reacting with H₂O₂, it is important to consider that enzymes involved in the conversion of superoxide to H₂O₂ may influence the amount of H₂O₂ in the cells (Buettnner, Ng, Wang, Rodgers, & Schafer, 2006). As DCF fluorescence is not specific for superoxide, I chose to conclude that increased DCF fluorescence was due to increased ROS levels and not increased superoxide levels.

Adventitial fibroblasts were cultured on glass cover slips in 35 mm dishes, following treatment with ET-1 and related chemicals, cells were treated with DCF-DA (5 μM) for 5-20 mins at room temperature in dim light. Treated adventitial fibroblasts on cover slips were then placed on a glass slide under a Nikon Eclipse 80i microscope. An excitation wavelength of 485 nm was used to visualize the excitation wavelength of 529 nm. Photomicrographs were taken and compared. Previous studies have used similar methods with cardiac fibroblasts (Cheng et al., 2003).

To quantify the photomicrographs obtained using DCF-DA, 10 representative cells from each sample were isolated and total fluorescence was taken (red, green, blue) from these highlighted regions were taken with parameters remaining constant for all
samples. The fluorescence from the 10 sample cells was combined to produce an average for the entire sample. The relative scale used for fluorescence ranged from 0 to 65,000. The program used to quantify the fluorescence was SimplePCI C-Imaging system.

The classical protocol for DCF utilizes duplicate catalase samples to quench all H$_2$O$_2$ related fluorescence. This quenching of fluorescence confirms that the fluorescence being observed is due to reactions with H$_2$O$_2$ and not due to redox-cycling (Dikalov et al., 2007). As later discussed in XI.2, this aspect of the DCF protocol was completed on a small number of samples in X.1.B and on NOX2 KO cells in X.2.B. Once catalase treatment demonstrated that a significant portion of the DCF fluorescence was due to a reaction with H$_2$O$_2$, it was decided that my resources could be better utilized in completing more experiments with antagonists and inhibitors rather than duplicating all treatments with catalase treatments. This aspect of the protocol has also been omitted by other studies in the field (Cheng et al., 2003; Price, Uras, Banks, & Ercal, 2006; Vejrazka, Micek, & Stipek, 2005).

VIII.9. siRNA mediated inhibition of NOX4

Adventitial fibroblasts were cultured to passage 3 in 6-well plates for protein isolation. Cells were grown initially in 10% FBS DMEM/F-12 medium, however, one day before siRNA treatment, cells will be washed 3 times with sterile PBS and 10% FBS antibiotic free DMEM/F-12 medium was added. Adventitial fibroblasts were grown to a subconfluence of 60-80% before transfection treatment could occur. For each transfection, siRNA duplex specific for mouse NOX4 was diluted in siRNA transfection medium. The siRNA transfection reagent was also diluted in siRNA transfection medium. These two solutions were gently mixed together and incubated at room temperature for
15-45 mins. Following the incubation, the solution of siRNA duplex, siRNA transfection reagent and transfection medium was diluted with transfection reagent and mixed gently. At this point, the target cells were washed with transfection medium once and the solution containing the siRNA was added to the target cells. The cells were then incubated for 5-7 hrs in the humidified atmosphere incubator. Following incubation, 1 ml of 20% FBS DMEM/F-12, with 2 times the normal antibiotic mixture medium was added to the cells without removing the transfection mixture. Cells were then incubated for another 18-24 hrs. At this point, the medium was aspirated and replaced with fresh 10% FBS DMEM/F-12 medium. Final treatment occurred 24-72 hrs after the addition of fresh medium.

This protocol was adapted from the siRNA Transfection Protocol from Santa Cruz Biotechnology.

**VIII.10. Statistical Analysis**

Data are expressed as mean ± SEM. Data analysis and graphic analysis were performed by SigmaStat 3.1 software. Comparison of groups was performed using one-way ANOVA with repeated measures, with Tukey’s comparisons post hoc. Statistical significance was accepted at p<0.05.
IX. Results

IX.1. Objective: To examine whether ET-1 administered exogenously increases ROS production

IX.1.A. Objective: ET-1-induced superoxide production measured by lucigenin enhanced chemiluminescence

First, adventitial fibroblasts were stimulated with 10 nM exogenous ET-1 to determine if ET-1 induces an increase in superoxide levels. Following treatment with ET-1, a gradual increase in superoxide production by adventitial fibroblasts was observed (Figure 3). Superoxide levels remained at near basal levels until the 240 mins (4 hrs) when a significant peak occurred (357 ± 96 milliU/mg protein/min). After 240 mins of ET-1 stimulation, superoxide levels had increased by roughly 5 times versus the basal levels (p<0.05). At 360 mins, superoxide production was still elevated versus baseline levels but diminished by approximately 25% compared to peak superoxide levels at 240 mins. At 360 mins, superoxide levels are statistically significantly raised versus control (273 ± 54 milliU/mg protein/min) (p<0.05).

These results indicate that ET-1-induced an increase in superoxide levels in a time-dependent manner. Additionally, it was determined that cells would be treated with ET-1 for 4 hrs for the remaining experiments in Objective 1, 2, 3B and 4B as this is when Objective 1.A demonstrates maximal levels of superoxide were present.
Figure 3: Superoxide levels in response to ET-1. Adventitial fibroblasts were treated with 10 nM ET-1 for the indicated times, followed by superoxide measurement by lucigenin superoxide as indicated in the methodology. Results are means ± SEM of 9 experiments, * = p<0.05 versus t=0.

IX.1.B. Objective: ET-1-induced increase in ROS levels measured by 2',7'-dichlorofluorescein (DCF)

Due to the limitations associated with lucigenin enhanced chemiluminescence, DCF fluorescence was used to verify the measurements taken by lucigenin enhanced chemiluminescence. Fluorescence using DCF was clear and different fluorescent intensities were visible between treatment groups (Figure 4). ET-1 stimulation for 4 hrs (15 421 ±2060 arbitrary fluorescence units (AFU)) doubled fluorescence compared to the control (8404 ± 461 AFU) (p<0.05). Duplicate samples of control (4703 AFU) and ET-1 treated (8571 ± 290 AFU) (p<0.05 versus ET-1 sample) with catalase showed a decreased fluorescence, suggesting that DCF was reacting with H2O2.
Figure 4: ROS levels in response to ET-1. Adventitial fibroblasts were pre-treated with or without catalase (300 U/mL) for 40 mins prior to being treated with or without 10 nM ET-1 for 4 hrs. This was followed by ROS measurement by DCF fluorescence as indicated in the methodology. A - Photomicrographs representative of 1-3 experiments. Magnification = 100x. B - Representative fluorescence intensity by DCF of 10 cells in each sample. Results are expressed as average fluorescence intensities ± SEM, * = p < 0.05 versus control, ** = p<0.05 versus ET-1.
IX.1.C. Objective: $\text{ET}_A$- receptor mediates ROS levels in adventitial fibroblasts

Next, I examined the effect of ET receptor antagonism on the ET-1-induced increase in ROS production. Two different antagonists of the ET receptors were used, BQ 123 is a competitive specific antagonist for the $\text{ET}_A$ receptor ($\text{IC}_{50} = 8.9 \text{ nM}$) and BQ 788 is a specific allosteric antagonist for the $\text{ET}_B$ receptor ($\text{IC}_{50} = 0.9 \text{ nM}$) (Okada & Nishikibe, 2002).

Due to the small sample sizes, the results that were with lucigenin chemiluminescence completed did not reach the statistical significance, however, comparisons had p-values less than 0.1. The results indicate that incubation of adventitial fibroblasts with the $\text{ET}_A$ receptor antagonist BQ 123 inhibited the increase in superoxide levels due to ET-1 stimulation (Figure 5) (p<0.1 versus ET-1 sample) (64 ± 27 milliU/mg protein/min versus 277 ± 47 milliU/mg protein/min). Additionally, there was a large contrast in the levels of ET-1-induced ROS versus the control group (78 ± 39 milliU/mg protein/min versus 277 ± 47 milliU/mg protein/min) (p<0.1). Similarly, the $\text{ET}_B$ receptor antagonist has no significant effect on ET-1-induced ROS levels (265 ± 76 milliU/mg protein/min) (p<0.1).

Due to the small sample sizes and statistically insignificant results with lucigenin chemiluminescence, lucigenin chemiluminescent results were verified by using the DCF fluorescence for ROS measurement (Figure 6). It was found that an $\text{ET}_A$ receptor antagonist inhibited ROS production in response to ET-1 (p<0.05 versus ET-1 sample) (9486 ± 476 AFU versus 15421 ± 2060 AFU). The $\text{ET}_B$ receptor antagonist, BQ 788, again had no effect on ET-1-induced ROS production and a statistically significant difference was seen between ET-1 + $\text{ET}_B$ receptor antagonist and the control samples
(p<0.05) (14727 ± 649 AFU). These results indicate that increased ROS levels due to ET-1 stimulation are mediated through the ET\textsubscript{A} receptor as antagonism of this receptor can inhibit the ET-1-induced raise in ROS.

Both the ET\textsubscript{A} and ET\textsubscript{B} receptor antagonists had no effect on ROS levels without ET-1 treatment when measured with lucigenin enhanced chemiluminescence or DCF fluorescence.

Additionally, one sample with a combination of both ET\textsubscript{A} and ET\textsubscript{B} receptor antagonists was completed with and without ET-1 treatment. The samples emitted fluorescence intensities of 9615 and 9631 AFU respectively, both of which are slightly higher than the emitted fluorescence from control samples (8404 ± 461 AFU) but less than fluorescence emitted by ET-1 samples (15421 ± 2060 AFU).
Figure 5: Effect of ET receptor antagonists on superoxide levels in response to ET-1 stimulation. Adventitial fibroblasts were pre-treated in the presence or absence of an ET$_A$ receptor antagonist (BQ 123) (1 μM) or an ET$_B$ receptor antagonist (BQ 788) (1 μM) for 40 mins prior to being treated with or without 10 nM ET-1 for 4 hrs. Treatment continued with antagonists during ET-1 treatment. This was followed by superoxide measurement by lucigenin enhanced chemiluminescence as indicated in the methodology. Results are means ± SEM. ‡ = p<0.1 versus control, † = p<0.1 versus ET-1 sample.
Figure 6: Effect of ET receptor antagonists on ROS levels in response to ET-1 stimulation. Adventitial fibroblasts were pre-treated in the presence or absence of an ET\textsubscript{A} receptor antagonist (BQ 123) (1 μM) and/or an ET\textsubscript{B} receptor antagonist (BQ 788) (1 μM) for 40 mins prior to being treated with or without 10 nM ET-1 for 4 hrs. Treatment continued with antagonists during ET-1 treatment. This was followed by ROS measurement by DCF fluorescence as indicated in the methodology. A - Photomicrographs representative of 3-5 experiments. Magnification = 100x. B - Representative fluorescence intensity by DCF of 10 cells in each sample. Results are expressed as average fluorescence intensities ± SEM. * = p<0.05 versus control, ** = p<0.05 versus ET-1 sample.
IX.2. Objective: To determine whether NOX2 contributes to ET-1-induced ROS production.

IX.2.A. Objective: To determine the importance of NAD(P)H oxidase using pharmacological inhibitors

The inhibition of NAD(P)H oxidase in adventitial fibroblasts by pharmacological inhibitors was subsequently examined. Adventitial fibroblasts were treated with NAD(P)H oxidase inhibitors apocynin (10 μM), an inhibitor of NAD(P)H oxidase cytosolic subunits and binding with membrane bound subunits, and DPI (10 μM), an unspecific flavoprotein inhibitor. Treatments of adventitial fibroblasts with both apocynin (11181 ± 164 AFU) and DPI (8567 ± 877 AFU) inhibited ET-1-induced elevated ROS levels (15421 ± 2060 AFU) by a significant margin (p<0.05) with ROS levels remaining near basal levels.

These results suggest that NAD(P)H oxidase is involved in the ET-1-induced increase in ROS levels.

Apocynin treatment without ET-1 lead to a small increase in ROS levels versus the control samples that was not statistically significant (10421 ± 584 AFU versus 8404 ± 461 AFU respectively)(p=0.44), with ET-1 + apocynin treatment producing average fluorescent intensities that were very similar (11181 ± 164 AFU)(p=0.11 versus control) to solely apocynin samples.
Figure 7: Effect of NAD(P)H oxidase inhibitors on ROS levels in response to ET-1 stimulation. Adventitial fibroblasts were pre-treated in the presence or absence of apocynin (10 μM) or DPI (10 μM) for 40 mins prior to being treated with or without 10 nM ET-1 for 4 hrs. Treatment continued with inhibitors during ET-1 treatment. This was followed by ROS measurement by DCF fluorescence as indicated in the methodology. A - Photomicrographs representative of 3-5 experiments. Magnification = 100x. B - Representative fluorescence intensity by DCF of 10 cells in each sample. Results are expressed as average fluorescence intensities ± SEM. * = p < 0.05 versus control, ** = p < 0.05 versus ET-1 sample. † = p = 0.11 versus control.
IX.2.B. Objective: To determine the importance of NAD(P)H oxidase using NOX2 knockout adventitial fibroblasts

Adventitial fibroblasts that lacked the NOX2 subunit of NAD(P)H oxidase were treated with ET-1 and ROS levels measured with DCF fluorescence. NOX2 KO adventitial fibroblasts lacked a significant ET-1-induced response, with there being no difference between ET-1 (7539 ± 996 AFU) and control samples (7472 ± 1688 AFU) (p=1). There was a statistically significant decrease between WT ET-1 (15421 ± 2060 AFU) samples and NOX2 KO ET-1 samples (p<0.05). Catalase treatment had no effect on ROS levels in either the control (7652 ± 556 AFU) (p=1) or ET-1 (8043 ± 176 AFU) (p=1) NOX2 KO samples (Figure 8).

The fluorescence emitted by NOX2 KO adventitial fibroblasts control group was less than the WT control sample of adventitial fibroblasts (7472 ± 1688 AFU versus 8404 ± 461 AFU), although not statistically significant (p=0.9).

These results with NOX2 KO mice imply that a significant portion of the rise in ROS levels associated with ET-1 stimulation is mediated by the NOX2 isoform of NAD(P)H oxidase.
Figure 8: ROS levels in response to ET-1 in WT and NOX2 KO adventitial fibroblasts. Data for wild-type adventitial fibroblasts is taken from Figure 4. WT and NOX2 KO adventitial fibroblasts were pre-treated with or without catalase (300 U/mL) for 40 mins prior to being treated with or without 10 nM ET-1 for 4 hrs. This was followed by ROS measurement by DCF fluorescence as indicated in the methodology. **A** - Photomicrographs representative of 1-3 experiments. Magnification = 100x. **B** - Representative fluorescence intensity by DCF of 10 cells in each sample. Results are expressed as average fluorescence intensities ± SEM (n=3 for NOX2 KO samples). * = p < 0.05 versus wild-type control, ** = p < 0.05 versus wild-type ET-1 sample.
X.3. Objective: To determine whether NOX4 contributes to an ET-1-induced increase in ROS levels

IX.3.A. Objective: To determine NOX4 expression

The change in expression of NOX4 protein due to ET-1 stimulation in adventitial fibroblasts was then completed. NOX4 is present in adventitial fibroblasts and ET-1 induces a gradual increase in NOX4 protein in a time dependant manner. The increase in NOX4 protein levels appears at the 4hr mark (p<0.05), peaks at the 6 hr mark, and gradually decreases through the remaining time. The maximum increase of NOX4 protein at 6 hrs was a 2.3 fold increase (p<0.05). Following this increase, the level retreated to a 2.1 fold increase at 8 hrs (p<0.05), a 1.43 fold increase at 12 hrs (p<0.05) and concluded at a 1.46 fold increase after 24 hrs (p<0.05).

IX.3.B. Objective: To determine the effect of siRNA for NOX4 on an ET-1-induced increase in superoxide levels

This objective was unsuccessful as the attempts to use siRNA to decrease NOX4 protein levels were unsuccessful (data not shown, n = 2).
Figure 9: Effect of ET-1 incubation on NOX4 protein levels. Adventitial fibroblasts were treated with 10 nM ET-1 for the indicated times, followed by protein level measurement by Western blot as indicated in the methodology. A – Representative blot of NOX4 and GAPDH protein levels assessed by Western blot. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to verify equal loading. B – Results of Western blot analysis (means ± SEM of 3 experiments). * = p<0.05 compared to control. kDa = kiloDalton

IX.4. Objective: To examine whether the ERK-MAPK signaling pathway is essential for ET-1-induced ROS production
IX.4.A. Objective: To examine the ratio of phospho-versus total ERK1/2

Assessment of the effect of ET-1 stimulation on phospho-ERK1/2 protein levels was then completed. Treatment with ET-1 lead to an immediate increase in the levels of phosphoERK1/2 protein in adventitial fibroblasts (Figure 10). The increase in phosphoERK1/2 occurred immediately following ET-1 stimulation, with phosphoERK1/2 protein levels raising \( \approx 2.5 \) times versus baseline levels after only 5 mins. This increase was not found to be statistically significant (p=0.063). The increase in phosphoERK1/2 then peaked after 10 mins of treatment with ET-1 (p<0.05 versus control) rising to a level \( \approx 3.3 \) times baseline levels. PhosphoERK1/2 then quickly returned to near baseline levels after 15 mins of ET-1 stimulation, not rising again at any other tested time point.
Figure 10: Effect of ET-1 stimulation on phosphoERK1/2 levels. Adventitial fibroblasts were treated with 10 nM ET-1 for the indicated times, followed by protein level measurement by Western blot as indicated in the methodology. A - Representative blot of phosphoERK1/2 and total ERK1/2 protein levels assessed by Western blot. Total ERK1/2 protein was used to verify equal loading. B - Results of Western blot analysis. (means ± SEM of 3 experiments) * = p<0.05 compared to control, † = p<0.063 versus control. kDa = kiloDalton
IX.4.B. Objective: ET-1-induced superoxide production is inhibited by MEK1/2 inhibitor, measured by lucigenin enhanced chemiluminescence

A MEK1/2 inhibitor was used to examine the role for the ERK1/2-MAPK pathway in the ET-1-induced increase in ROS levels. A MEK1/2 inhibitor is useful as MEK1/2 is upstream of ERK1/2. When adventitial fibroblasts were treated with both ET-1 and a MEK1/2 inhibitor PD 98059 (31 ± 20 milliU/mg protein/min), no increase in superoxide levels were observed versus ET-1 (246 ± 50 milliU/mg protein/min) (p<0.05 versus ET-1) (Figure 11). In contrast, a negative analogue of the MEK 1/2 inhibitor, SB 202474, did not prevent an ET-1-induced superoxide response (322 ± 103 milliU/mg protein/min), with the results narrowly being statistically insignificant (p<0.1 versus control). Both ET-1 and ET-1 + the MEK 1/2 inhibitor negative analogue samples saw increases in superoxide levels that were 5-6 times that of baseline levels. These increases are consistent with those observed in the previous objectives that utilized lucigenin enhanced chemiluminescence to measure an increase superoxide levels due to ET-1 stimulation (Objective 1.A & 1.C).

Treatment with solely the MEK1/2 inhibitor (54 ± 36 milliU/mg protein/min) (p=1) or its negative analogue (28 ± 0 milliU/mg protein/min) (p=1) had no effect in superoxide production in cells without ET-1 treatment.

The results from this experiment indicate that the ERK1/2 pathway was a primary signaling pathway utilized in raising superoxide levels due to ET-1 stimulation.
Figure 11: Effect of MEK1/2 inhibitors on superoxide levels in response to ET-1 stimulation. Adventitial fibroblasts were pre-treated in the presence or absence of a MEK1/2 inhibitor (PD 98059) (10 μM), an upstream regulator of ERK1/2, and an inactive analogue of PD 98059 SB 202474 (10 μM) for 40 mins prior to being treated with or without 10 nM ET-1 for 4 hrs. Treatment continued with inhibitors during ET-1 treatment. This was followed by superoxide measurement by lucigenin enhanced chemiluminescence as indicated in the methodology. Results are means ± SEM, * = p<0.05 versus control, † = p<0.1 versus control, ** = p<0.05 versus ET-1.
IX.5. Objective: To determine whether Ang II contributes to superoxide production and determine if AT receptors and ET receptor mediate superoxide production

IX.5.A. Objective: To examine whether Ang II contributes to superoxide production

Next, adventitial fibroblasts were stimulated with 100 nM Ang II to determine if Ang II induces an increase in superoxide levels. Increased levels of superoxide were detected after the cells were incubated with Ang II for 30 mins (258 ± 11 milliU/mg protein/min) (p<0.05) with the level continuing to increase after 60 mins of incubation with Ang II (337 ± 28 milliU/mg protein/min)(p<0.05) (Figure 12). The first spike in superoxide levels was detected after 90 mins of Ang II stimulation (441 ± 61 milliU/mg protein/min), with levels increasing ≈4 fold versus control samples. The second peak was detected after 360 mins of Ang II incubation (346 ± 27 milliU/mg protein/min) (p<0.05) after levels had decreased at the 120 (205 ± 9 milliU/mg protein/min) and 240 min (247 ± 26 milliU/mg protein/min) mark, although both were still significantly elevated versus control cells (p<0.05). The increase in superoxide production after 6 hrs of Ang II treatment was ≈3 fold versus control samples.

These results demonstrate that Ang II induced an increase in superoxide levels in a biphasic manner with the largest amount of superoxide being observed at 90 and 360 mins.
Figure 12: Superoxide levels in response to Ang II. Adventitial fibroblasts were treated with 100 nM Ang II for the indicated times, followed by superoxide measurement by lucigenin superoxide as indicated in the methodology. Results are means ± SEM of 4 experiments, * = p<0.05 versus matching control times.
**IX.5.B. Objective:** To examine the role of \( \text{AT}_1 \) and \( \text{AT}_2 \) receptors in mediating Ang II-induced superoxide production

The next aspect of the study examine what role the \( \text{AT}_1 \) and \( \text{AT}_2 \) receptors have in raising superoxide levels due to Ang II stimulation. Adventitial fibroblasts were treated with 100 nM Ang II and in the presence or absence or a selective \( \text{AT}_1 \) receptor antagonist, Losartan (100 \( \mu \)M), or a selective \( \text{AT}_2 \) receptor antagonist, PD 123319 (100 \( \mu \)M). Increased levels of superoxide induced by Ang II were present after 6 hrs (346 ± 28 milliU/mg protein/min) and were completely inhibited with Losartan, the \( \text{AT}_1 \) receptor antagonist (100 \( \mu \)M) (36 ± 8 milliU/mg protein/min) (Figure 13). Losartan also decreased superoxide production in the control group (39 ± 9 milliU/mg protein/min) \((p<0.05)\), indicating that a portion of basal superoxide levels are due to the \( \text{AT}_1 \) receptor. Additionally, treatment of cells with a selective \( \text{AT}_2 \) receptor antagonist PD 123319 did not inhibit the Ang II induced increase in superoxide production (378 ± 12. milliU/mg protein/min).

The increase in superoxide production that was observed due to Ang II following 6 hrs of stimulation was \( \approx 3 \) fold in comparison to the control group.

By demonstrating inhibition of Ang II induced increase superoxide levels by antagonism of the \( \text{AT}_1 \) receptor, these results illustrate that the \( \text{AT}_1 \) receptor mediates a majority of the rise in superoxide levels associated with Ang II treatment following 6 hrs of Ang II stimulation.
Figure 13: Effect of AT receptor antagonists on superoxide levels in response to Ang II stimulation. Adventitial fibroblasts were pre-treated in the presence or absence of an AT<sub>1</sub> receptor antagonist, Losartan (100 µM) or an AT<sub>2</sub> receptor antagonist, PD 123319 (100 µM) for 40 mins prior to being treated with or without 100 nM Ang II for 6 hrs. Treatment continued with antagonists during Ang II treatment. This was followed by superoxide measurement by lucigenin enhanced chemiluminescence as indicated in the methodology. Results are means ± SEM of 4 experiments, * = p<0.05 versus control.
IX.5.C. Objective: To determine the role of ET\textsubscript{A} and ET\textsubscript{B} receptors in mediating Ang II-induced superoxide production

Finally, the role of ET receptors in the Ang II induced rise in superoxide levels was examined. The selective ET\textsubscript{A} and ET\textsubscript{B} receptor antagonists BQ 123 (1 \( \mu \)M) and BQ 788 (1 \( \mu \)M) respectively were again used. Treatment of the cells with the selective ET\textsubscript{A} receptor antagonist was able to inhibit the Ang II induced increase in superoxide levels observed following 6 hrs of stimulation (73 ± 14 milliU/mg protein/min versus 347 ± 27 milliU/mg protein/min). Additionally, an ET\textsubscript{B} receptor antagonist was not able to inhibit Ang II induced superoxide at the 6 hr mark (368 ± 8 milliU/mg protein/min versus 110 ± 15 milliU/mg protein/min)(p<0.05 versus control). The superoxide increase observed with both ET-1 and ET-1 + an ET\textsubscript{B} receptor antagonist samples was ≈3.5 versus baseline levels.

Antagonism of either receptor in the absence of Ang II had no effect superoxide levels (BQ 123, 127 ±9 milliU/mg protein/min, BQ 788, 126 ± 9 milliU/mg protein/min)

These results indicate that the Ang II induced increase in superoxide is mediated primarily through the ET\textsubscript{A} receptor after 6 hrs.
Figure 14: Effect of ET receptor antagonists on superoxide levels in response to Ang II stimulation. Adventitial fibroblasts were pre-treated in the presence or absence of an ET$_{A}$ receptor antagonist (BQ 123) (1 µM) or an ET$_{B}$ receptor antagonist (BQ 788) (1 µM) for 40 mins prior to being treated with or without 100 nM Ang II for 6 hrs. Treatment continued with antagonists during Ang II treatment. This was followed by superoxide measurement by lucigenin enhanced chemiluminescence as indicated in the methodology. Results are means ± SEM of 4 experiments, * = p<0.05 versus control.
X. Discussion

This project has established the ROS response to both ET-1 and Ang II stimulation in adventitial fibroblasts. Five different aspects of these responses were documented. (1) ET-1 stimulates ROS production via an ET\textsubscript{A} receptor mediated pathway. (2) The enzymatic source of ET-1 is predominately due to the NOX2 isoform of the NAD(P)H oxidase enzyme. (3) The protein level of the NOX4 subunit of the NAD(P)H oxidase is elevated by ET-1 stimulation although its role in ROS production is yet to be demonstrated. (4) ET-1 stimulates ROS production through an ERK1/2 MAPK mediated pathway. (5) Ang II stimulates ROS production in a biphasic manner, with the \textit{AT\textsubscript{1}} and ET\textsubscript{A} receptors being responsible for mediating the second phase.

X.1. Characterization of adventitial fibroblasts:

The primary culture method used in the present study has been published previously (Chamseddine & Miller, Jr., 2003; Gao et al., 2003; Guo et al., 2006; Haurani et al., 2008; Jenkins et al., 2007; Pagano et al., 1998; An et al., 2006; An et al., 2007). Due to the absence of a cell-specific marker for adventitial fibroblasts, we characterized cultured cells by eliminating other cell types. The results showed that the cells cultured under the conditions described in this thesis expressed the non-specific cell marker vimentin, but did not express specific cells markers of ECs, VSMCs and leukocytes (An et al., 2006).

X.2. ET-1 stimulates ROS production:

My results demonstrate for the first time that ET-1 increases ROS production in vascular adventitial fibroblasts. ET-1 increases superoxide production in a time-dependent manner. ET-1-induced a gradual increase in superoxide levels in the first 2 hrs
of treatment, followed by an elevation at 4 hrs of ET-1 treatment (Figure 3). This time frame for increased levels of superoxide is similar to that observed by Laplante et al. (2005) in VSMCs. In addition, Cheng et al. (2003) also reported that ET-1-induced ROS production in rat cardiac fibroblasts; however, the increase of ROS in cardiac fibroblasts was rapid, plateauing after only 30 mins of ET-1 stimulation. In ECs, NOX2 mRNA levels and activity peaked following 60 and 90 mins of ET-1 stimulation respectively (Duerrschmidt et al., 2000).

Due to limitations later discussed (XI.8.B.) and the variations I experienced with lucigenin enhanced chemiluminescence readings, I also measured ROS production using DCF fluorescence. Consistent with the superoxide data measured by lucigenin enhanced chemiluminescence, the results obtained using DCF clearly show that ET-1 increased ROS production after 4 hrs of ET-1 stimulation (Figure 4).

One component of the DCF assay as previously discussed (IX.8.B) involves duplicating samples with the enzyme catalase. In my study, this aspect of the DCF assay was only completed for Objectives 1.B and 2.B. The results that were produced with catalase treatment demonstrate a lower level of fluorescence (Figure 4 and Figure 8), however, catalase did not quench all fluorescence. Potential reasons for the remaining fluorescence include autofluorescence due to collagen (Gareau, Bargo, Horton, & Jacques, 2004), fluorescence due to non-specific binding of DCF or the remaining intracellular H$_2$O$_2$ that was not removed as catalase was restricted to the extracellular region (Dikalov et al., 2007). Additionally, redox reactions with the activated and unactivated forms of DCF can lead to an increase in ROS levels and decrease antioxidant capacity. In the literature, several in vitro studies that utilized DCF-DA excluded the
catalase aspect of the assay (Cheng et al., 2003; Price et al., 2006; Vejrazka et al., 2005), suggesting that results lacking duplicate samples with catalase are accepted within the scientific community. Due to limited resources, particularly related to the number of WT adventitial fibroblasts that were available for my experiments, it was determined that catalase duplication would be completed on control and ET-1 treatments in WT adventitial fibroblasts first and the results would be evaluated. When catalase treatments for these samples (Figure 4) decreased fluorescence, it was determined that a significant portion of observed DCF fluorescence was due to reactions with H₂O₂. After concluding that my DCF fluorescence was primarily due to ROS, I decided that my remaining resources would be better utilized in completing more trials with inhibitors or antagonists instead of duplicating my samples with catalase.

My results demonstrate that the ETₐ receptor mediates ET-1-induced ROS. ET-1-induced superoxide production measured by lucigenin enhanced chemiluminescence was decreased by an ETₐ receptor antagonist (Figure 5). Similarly, the ROS production in response to ET-1 measured by DCF was also reduced by an ETₐ receptor antagonist (Figure 6) and combination treatment with both an ETₐ and ETₐ receptor antagonist. These results were comparable with previous findings in VSMCs (Laplante et al., 2005), cardiac fibroblasts (Cheng et al., 2003) and in the DOCA-salt hypertensive model, a model known for high ET-1 levels (Callera et al., 2003).

Despite the apparent role of the ETₐ receptor, the contribution of ETₐ receptors in ROS production is controversial. My results show that ET-1-induced ROS was abolished by an ETₐ receptor antagonist but not inhibited by the ETₐ antagonist, excluding the ETₐ receptor in mediating ET-1-induced ROS. My results are comparable to those produced
by Laplante et al. (2005) in VSMCs and Cheng et al. (2003) in cardiac fibroblasts. Contrary to our findings, Loomis et al. (2005) treated explanted aorta (aorta removed from body and cleaned of connective and adipose tissue) with ET-1 but were not able to inhibit ET-1-induced superoxide production solely with an ET\textsubscript{A} receptor antagonist. It required a combination ET\textsubscript{A} + ET\textsubscript{B} receptor antagonist to stop the increase in superoxide production associated with ET-1. Furthermore, an ET\textsubscript{B} receptor antagonist inhibited a significant amount of ET-1-induced superoxide production, suggesting that ET\textsubscript{B} receptors also mediate ET-1-induced superoxide.

**X.3. NOX2 and the ET-1 Response:**

NAD(P)H oxidase produces a majority of ROS generated in the vessel wall (Touyz et al., 2004a) with large quantities of this enzyme being located in the adventitia (Wang et al., 1998). The significance of NAD(P)H oxidase was first demonstrated in my study by pharmacological inhibitors. DPI and apocynin, two NAD(P)H oxidase inhibitors, were able to inhibit ET-1-induced ROS production (Figure 7). However, the interpretation of my results may be limited due to the nonspecificity of the inhibitors. DPI is an unspecific flavin oxidant, which limits electron transfer and disrupts many superoxide producing enzymes in addition to NAD(P)H oxidase (Heumuller et al., 2008). Compared with DPI, apocynin was considered a more specific inhibitor as it would prevent the assembly of the membrane bound subunits of the NAD(P)H oxidase enzyme (NOX, p22\textsuperscript{phox}) with the cytosolic subunits (p47\textsuperscript{phox}, p67\textsuperscript{phox}), particularly in phagocytic cells (Vejrazka et al., 2005; Bedard et al., 2007). However, recent research by Heumuller et al. (2008) has challenged the idea that apocynin limits the function of NAD(P)H oxidase in non-phagocytic cells. Heumuller et al. (2008) demonstrate that superoxide
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production in human embryonic kidney cells transfected with NOX1, 2 & 4 and p47\textsuperscript{phox} and p67\textsuperscript{phox} was not affected by treatment with apocynin. Additionally, when examining the effect of apocynin on other superoxide generating systems, it was noted that apocynin decreased superoxide levels, suggesting that apocynin decreases superoxide levels through an antioxidant capacity in vascular cells.

To add to the complexity of the matter, my treatment solely with apocynin produced a small but not significant rise in superoxide levels (Figure 7). This characteristic increase in superoxide levels due to apocynin treatment has previously been documented in vascular fibroblasts by Vejrazka et al. (2005). Vejrazka et al. (2005) presented a doubling in DCF fluorescence due to apocynin treatment which is significantly more than the \approx 24\% increase I observed.

Due to the nonspecific effects of the inhibitors DPI and apocynin, the role of NOX2 was tested in adventitial fibroblasts isolated from mice that lack the NOX2 isoform.

The lack of superoxide production observed in NOX2 KO adventitial fibroblasts (Figure 8) suggests a prominent role for NOX2 derived ROS in ET-1-induced ROS production. The ET-1/NOX2 pathway may be similar to the initial phase of superoxide production observed due to Ang II stimulation in adventitial fibroblasts (Figure 12 at 1.5 hrs) (An et al., 2007). As direct measurement of NOX2 protein levels was not completed, it remains unclear as to whether this increase in ROS production due to ET-1 from NOX2 is due to a de-novo protein synthesis or due assembly and activation of existing NAD(P)H oxidases.
In other cell types and animal models, ET-1 stimulation has been linked with many other ROS producing enzymes. In the DOCA-salt hypertensive rats, research is demonstrating that xanthine oxidase and mitochondrial sources are significantly involved in ROS production (Callera et al., 2006; Viel et al., 2008). In VSMCs, both mitochondrial sources (Touyz et al., 2004b) and NAD(P)H oxidase have been implicated (Laplante et al., 2005). In extracted aorta, ET-1-induced ROS production was linked to NAD(P)H oxidase and NOS uncoupling (Loomis et al., 2005). The research by Laplante et al. (2005) has shown that ET-1 mediates Ang II-induced ROS in Ang II-dependent hypertension as a combination ET_A + ET_B receptor blocker significantly decreased superoxide levels (Laplante et al., 2005). Previous research has also shown that a significant portion of superoxide in Ang II-dependent hypertension is due to NOX2 from the adventitia (Wang et al., 2001). This combination of results from Laplante et al. (2005) and Wang et al. (2001) support the results I produced implicating NOX2 in ET-1-induced ROS production in adventitial fibroblasts.

Pharmacological inhibitors and a genetic KO model established the role for the NOX2 isoform of NAD(P)H oxidase in ET-1-induced ROS production. In adventitial fibroblasts, NOX2 plays a significant role in ROS production associated with ET-1 stimulation.

X.4. NOX4 in adventitial fibroblasts and the ET-1 Response

The NOX4 gene is known to be upregulated in rat adventitial fibroblasts (Guo et al., 2006) and involved in increased ROS production in cardiac fibroblasts (Cucoranu et al., 2005) with treatment of TGF-β. In human cardiac fibroblasts, NOX4 mRNA increased following H2O2, diacylglycerols, and arachidonic acid stimulation (Colston, de
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In VSMCs, the ET_A receptor has been shown to be involved in a rise in NOX4 protein levels due to 6 hrs of Ang II stimulation. An ET_A receptor antagonist was able to inhibit this increase suggesting a role for ET-1 in the rise of NOX4 protein levels (Laplante et al., 2005). As with the results of Laplante et al. (2005), my results demonstrate an increase in NOX4 protein levels. With ET-1 treatment there is an increase of NOX4 protein peaking at the 6 hr mark (2.3 fold versus control), then gradually decreasing (Figure 10). siRNA was to be used to determine the impact of NOX4 on superoxide levels as has been completed previously (Cucoranu et al., 2005). Unfortunately, with the protocol and reagents that were used, no decrease in NOX4 protein levels at 6 hrs was observed (data not shown). As the siRNA purchased from Santa Cruz Biotechnology is specific for 3 different regions of mice NOX4 mRNA, it is likely that the combination of transfection reagent and transfection medium was not successfully in allowing the siRNA into the cell. In the studies in which NOX4 siRNA has been successful in adventitial fibroblasts, Invitrogen’s lipofectamine has been used as the transfection reagent (Haurani et al., 2008).

NOX4 is a NAD(P)H oxidase isoform that contributes to ROS production in cardiac fibroblasts (Cucoranu et al., 2005) and mesangial cells (Gorin et al., 2003). The data collected in NOX2 KO adventitial fibroblasts suggests that NOX4 derived ROS mediates a minor portion of ET-1-induced ROS as when NOX2 is absent, no significant increase in ROS is observed (Figure 8). Additionally, the increase in NOX4 protein in adventitial fibroblasts due to ET-1 stimulation (Figure 9) is not consistent with the timeline for ET-1-induced ROS production (Figure 3). Superoxide production peaks after 4 hrs of ET-1 treatment where as NOX4 protein levels peak after 6 hrs of ET-1
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treatment. NOX4 derived ROS may be more significant after 6 hrs of ET-1 stimulation as ROS levels, although lower than after 4 hrs of ET-1 stimulation, are still significantly higher than basal levels. It is also unlikely that NOX4 would significantly contribute to the later stages of ET-1-induced ROS as NOX4 protein levels are only increased by ≈1.4 after 12 and 24 hrs of ET-1 stimulation.

At this point, it is not clear as to what the role of the NOX4 increase is. One possibility is related to the conversion of the adventitial fibroblasts to myofibroblasts. It has previously been documented that NOX4 is involved in this conversion in cardiac fibroblasts due to TGF-β stimulation and a 16-fold increase in NOX4 mRNA expression was observed (Cucoranu et al., 2005). That 16-fold increase in NOX4 mRNA expression is much greater than the 2.3-fold protein increase I observed in adventitial fibroblasts (Figure 9). In lung adventitial fibroblasts, the conversion from adventitial fibroblast to myofibroblast characterized by the produced of α-SM actin was observed following 4 hrs of ET-1 treatment (Shi-Wen et al., 2004), coinciding with my peak in ROS production but not with the peak in NOX4 protein levels.

From the results I produced, it is unlikely that NOX4 is the primary mediator for the ET-1-induced conversion of adventitial fibroblasts to myofibroblasts or the primary enzyme responsible for maintaining the myofibroblasts phenotype due to inconsistencies with the time frame that NOX4 protein levels are elevated. A recent study of NOX4 in adventitial fibroblasts presented a contrasting conclusion with regards to Ang II induced conversion of adventitial fibroblasts. NOX4 was found to be responsible for the migration associated with the conversion of adventitial fibroblasts to myofibroblasts due to Ang II stimulation (Haurani et al., 2008). Decreased migration was observed when
H$_2$O$_2$ levels and NOX4 mRNA decreased. This decrease in migration led to the conclusion that NOX4 is involved in the conversion or maintenance of adventitial fibroblasts to myofibroblasts.

The conversion of adventitial fibroblasts to myofibroblasts and the maintenance of this phenotype is significant due to the contractile proteins such as $\alpha$-SM-actin that develop during this conversion (Garanich, Mathura, Shi, & Tarbell, 2007; Sartore et al., 2001). The contractile proteins that develop allow myofibroblasts to become motile and migrate within the vessel. This motility has been shown to be important in the development of neointimal hyperplasia (Li et al., 2000; Li et al., 2002b; Scott et al., 1996; Shi et al., 1996; Siow et al., 2003). It has also been hypothesized that the conversion of adventitial fibroblasts to myofibroblasts leads to an increase in the number of growth factors and cytokines that are released by the cell (Plekhanova et al., 2006). As a majority of knowledge surrounding myofibroblasts is derived from research in wound healing, the details of myofibroblast activity in the vasculature have not been explored. It is due to the myofibroblast role in wound healing that the hypothesis of vessel repair being mediated by the adventitia has received support (Maiellaro et al., 2007).

**X.5. ERK1/2 MAPK in the ET-1 Response**

With ET-1 stimulation of adventitial fibroblasts in culture, there was a significant increase in phosphorylation of the ERK1/2 portion of the MAPK pathway. The increase in phospho-ERK1/2 levels was $\approx$3.3 fold greater than the control levels and peaked 10 mins following ET-1 stimulation (Figure 10). When ERK1/2 was inhibited the ET-1-induced rise in superoxide production was absent (Figure 11). The results observed in
adventitial fibroblasts show an ERK1/2 dependent pathway in the production of superoxide due to ET-1

The quick peak of phosphorylation of ERK is consistent with that found in VSMCs (Touyz et al., 2004b; Daou et al., 2004; Yogi et al., 2007) but differing from rat cardiac fibroblasts as in rat cardiac fibroblasts, phospho-ERK1/2 levels spiked following 30 mins of ET-1 treatment (Cheng et al., 2003).

Studies involving ERK1/2 and ET-1 treatment have come to differing conclusions as to whether ROS production is upstream or downstream of ERK1/2. In VSMCs, increased ERK1/2 levels associated with ET-1 stimulations do not change when antioxidants are present (Touyz et al., 2004b), suggesting ERK1/2 could be upstream of ROS production. Other studies in cardiac fibroblasts (Cheng et al., 2003) and VSMCs (Daou et al., 2004; Laplante et al., 2006) suggest that ERK1/2 is downstream. In these studies suggesting ERK1/2 is downstream of superoxide production, ERK1/2 inhibitors had no effect on superoxide production (Laplante et al., 2006) and antioxidant treatment decreased phospho-ERK1/2 levels (Cheng et al., 2003; Daou et al., 2004).

The results from Objective 5 may not completely illustrate the signaling pathway involved in ET-1-induced superoxide production. Due to the extended time period observed between ET-1 stimulation and peak levels of ROS, ERK1/2 may become activated at other points that my protocol missed. Only 7 time points were examined during this experiment, a majority immediately following ET-1 stimulation. The focus on this time frame is due to the previous research in the field (Cheng et al., 2003; Daou et al., 2004; Laplante et al., 2006; Touyz et al., 2004b). By concentrating on the time immediately following ET-1 stimulation, there is a large portion of time where phospho-
ERK1/2 levels were not examined. An increase in phospho-ERK1/2 levels only requires a few mins to spike as demonstrated by Figure 10, and may have returned to baseline levels without being detected.

**X.6. Contribution of AT and ET receptors on Ang II induced ROS production**

Prior studies have suggested increased production of ROS due to Ang II in an ex vivo setting (Wang et al., 1998) and via particulate fraction from adventitial fibroblasts (Pagano et al., 1998). With treatment of Ang II to cultured adventitial fibroblasts, I demonstrate for the first time that superoxide production occurs in 2 phases, with the initial peak occurring at 1.5 hrs, the other after 6 hrs of Ang II treatment (Figure 12). Use of receptor antagonists demonstrates that the second peak that occurs after 6 hrs of Ang II treatment can be inhibited by both an AT₁ receptor antagonist (Figure 13) and an ETₐ receptor antagonist (Figure 14).

An et al. (2006) show an increase of preproET-1 occurs 1.5 hr following Ang II treatment in adventitial fibroblasts. Further research demonstrates that antioxidants or NAD(P)H oxidase inhibitors can decrease levels of preproET-1 significantly (An et al., 2007). An et al. (2007) demonstrated that the ET-1 produced was functional and contributed to an increase in the ECM protein collagen. An et al. (2007) concluded that ET-1 is released by cultured adventitial fibroblasts and that this ET-1 release is induced by ROS production due Ang II stimulation. These findings lead us to the conclusion that the initial phase of Ang II induced superoxide production (Figure 12) leads to ET-1 being synthesized and released by adventitial fibroblasts. This ET-1 then leads to increased levels of superoxide 4 hrs subsequent to its release. By combining my results with the known literature, I have concluded that treatment with the AT₁ receptor antagonist could
not only inhibit the second phase of superoxide production, but also the first. Treatment with the ET\textsubscript{A} receptor antagonist would only be successful in inhibiting the secondary phase of superoxide production.

Research in VSMCs supports the theory that Ang II-induced ET-1 production results in elevated levels of superoxide. Similar experiments demonstrate that superoxide levels due to Ang II treatment could be inhibited by an ET\textsubscript{A} receptor antagonist (Laplante et al., 2005). Ang II-induced ET-1 release in VSMCs is known to occur due to Ang II stimulation (Sung, Arleth, Storer, & Ohlstein, 1994). My results indicate that similar Ang II-induced ET-1 release and ROS pathways occur in both VSMCs and adventitial fibroblasts.

Treatment with an AT\textsubscript{1} receptor antagonist but without Ang II stimulation was able to significantly decrease superoxide levels (Figure 13). These results were surprising as they suggest that \textit{in vitro} adventitial fibroblasts are able to synthesize Ang II, a characteristic that has not been documented in the literature. Research has demonstrated that entire vessels are able to produce Ang II from circulating angiotensinogen (Brasier, Recinos, III, & Eledrisi, 2002) and high levels of angiotensinogen mRNA have been found in the adventitia following balloon injury (Rakugi, Jacob, Krieger, Ingelfinger, & Pratt, 1993). Other than the production of angiotensinogen, no research has demonstrated the presence of renin or ACE enzyme in the adventitia or in cultured adventitial fibroblasts. These two enzymes would be required to covert angiotensinogen to the active Ang II. There is also no evidence that Losartan, the AT\textsubscript{1} receptor antagonist, has antioxidant characteristics. At present, I am unable to rule out the possibility that adventitial fibroblasts are able to produce a basal
level of Ang II in culture demonstrated by inhibition of superoxide production by an AT₁ receptor antagonist.

X.7. ET Receptor Antagonists

One inconsistency within this thesis is the working concentration used for the ET₉ receptor antagonist BQ 788. These variations are due to some questioning regarding its effectiveness as a selective ET₉ receptor antagonist due to a low ETₐ receptor IC₅₀. In the study by Okada & Nishikibe (2002), BQ 788 had an IC₅₀ of 280 nM for the ETₐ receptor in porcine coronary artery smooth muscle (pCASAM) versus the ET₉ receptor IC₅₀ of ≈1 nM in porcine cerebellar membranes and human Giradi cardiac cells (Okada et al., 2002). A review of the above experiments shows that Figures 5 and 14 utilized concentrations of BQ 788 almost tripling the pCASAM IC₅₀ value for the ETₐ receptor (1 μM). The impact associated with the use of BQ 788 at 1 μM remains unclear.

Theoretically, during my experiments that used BQ 788 at 1 μM, a significant amount of ETₐ receptors as well as ET₉ receptors were antagonized and thus inhibited, yet no effects of ETₐ inhibition were observed. Okada & Nishikibe (2002) also list in vitro studies in which 1 μM treatment of BQ 788 was not sufficient in obstructing ETₐ receptor responses. One potential reason for the lack of inhibition could be due to a difference in IC₅₀ values for VSMCs and adventitial fibroblasts. Okada & Nishikibe (2002) list another cell type, the human neuroblastoma cell that has a higher IC₅₀ for the ETₐ receptor than pCASAM at 1300 nM. Despite this information regarding the ETₐ receptor and BQ 788, a majority of in vitro studies continue to utilize the 1 μM concentration and state that BQ 788 is a selective ET₉ receptor antagonist (Prefontaine, Calderone, & Dupuis, 2008; Zeng, Zhou, Yao, O'Rourke, & Sun, 2008). Even with 1 μM
being the standard concentration *in vitro*, once the study by Okada & Nishikibe (2002) was brought to the researcher's attention, the remaining experiment (Figure 6) was conducted using a concentration reduced ten-fold (100 nM).

**Issues related to the ET receptor are not isolated to the ET<sub>B</sub> receptor antagonist BQ 788 as issues with the ET<sub>A</sub> receptor antagonists have been identified in the literature as well.** A study by Harada et al. (2002) provided evidence that suggests that ET receptors from the pituitary gland may form a heterodimer complex. With the hypothesized ET<sub>A/B</sub> receptor complex, it was concluded that the ET<sub>A</sub> section of the heterodimer is responsible for the recognition of ET-1 and that the ET<sub>B</sub> receptor is unable to recognition ET-1 without the aid of ET<sub>A</sub> receptor (Harada, Himeno, Shigematsu, Sumikawa, & Niwa, 2002). If the hypothesis of Harada et al. (2002) is correct, treatment of an ET<sub>A</sub> receptor antagonist would have the characteristics of a dual ET receptor antagonist as the ET<sub>B</sub> receptor would be unable to identify ET-1. The conclusion of Harada et al. (2002) is challenged in VSMCs by studies in which ET<sub>B</sub> receptor activity continues to occur despite ET<sub>A</sub> antagonism, such as in the work done looking at ET<sub>B</sub> receptor dependent ET-1 vasoconstriction (Hersch, Huang, Grider, & Murthy, 2004; Inui et al., 1999). Other vascular related studies that have utilized ET<sub>A</sub> antagonism have continued to state that ET<sub>A</sub> receptor antagonists inhibit only ET<sub>A</sub> receptors and researchers have made their conclusions accordingly (Callera et al., 2003; Laplante et al., 2005; Li et al., 2003a). **My results with ET antagonism of both ET-1 (Figure 5) and Ang II (Figure 14) stimulation do not disprove the hypothesis of Harada et al. (2002).** Adventitial fibroblast may have all three types of receptors, independent ET<sub>A</sub>, independent ET<sub>B</sub> and ET<sub>A/B</sub> complex. However, I was not able to confirm the hypothesis
and will not be able to do so until I (1) immunoprecipitate one of the receptors and blot for the other, i.e. $ET_A$ and blot for the $ET_B$ receptor; (2) compare the results with total protein levels of $ET_A$ and $ET_B$ via Western blot. In light of the research that contradicts their hypothesis, my conclusions were based on the $ET_B$ receptor being able to independently identify ET-1 without requiring the $ET_A$ receptor.

**X.8. Limitations of Methods**

With the use of any experimental procedure or method, there exist both benefits and limitations. For the purpose of disclosure regarding the present study, it is prudent to highlight some benefits of the chosen methods as well as some limitations of the study.

**X.8.A. Characterization of isolated adventitial fibroblasts**

The initial issue for the proposed study to produce relevant results is to ensure that the cells that are cultured are in fact adventitial fibroblasts. Unlike other cell types such as ECs, VSMCs or human fibroblasts, mouse adventitial fibroblast cells lack a specific cell marker to conclusively identify them. Due to the lack of specific cell marker, my laboratory opted to identify through exclusion. This identification through exclusion was accomplished by testing the cultured cells with specific cell markers for cell lines that may contaminant my culture.

In the previous study in our laboratory (An et al., 2006), both mRNA and immunocytochemical staining demonstrated a very low or lack of expression for cell markers specific for differentiated and non-differentiated VSMC, ECs and leukocytes, suggesting that the culture is predominately adventitial fibroblasts. However, due to a lack of a fibroblast cell marker, there remains the possibility of contamination of other cell types, such as pericytes, that lack a specific cell marker as well. Nevertheless, the
possibility of pericytes contaminating our culture is unlikely to occur with our culture protocol as high levels of pericytes have only been found during the embryonic stage with a dramatic decrease is found in adult mice. Although pericytes are still present in adult mice, the levels are considered insignificant (Cho, Kozasa, Bondjers, Betsholtz, & Kehrl, 2003). As our culture protocol used adult mice, it is unlikely that a significant number of pericytes would be present in our culture.

Although there remains no conclusive method to demonstrate the presence of adventitial fibroblasts, the use of cultured adventitial fibroblasts is becoming more frequent as several studies have utilized this model (Garanich et al., 2007; Guo et al., 2006; Kim et al., 1999; Pagano et al., 1997; Pagano et al., 1998; Shen et al., 2006; An et al., 2006; An et al., 2007; Haurani et al., 2008), and a recent study was solely dedicated to the proper protocols specifically for adventitial fibroblasts (Jenkins et al., 2007).

X.8.B. Reactive oxygen species measurement

Several techniques exist for the testing of ROS, and all have potential weaknesses. ROS are difficult compounds to study due to both their short life span and their high reactivity, however lucigenin enhanced chemiluminescence has been proven to be one of the most reliable methods employed (Dikalov et al., 2007). The primary weakness of the technique lies in the ability of lucigenin to partake in redox-cycling, causing a signal to occur when no superoxide is present. However, concentrations of lucigenin will remain below the known concentrations at which redox-cycling is observed in vascular tissue (Dikalov et al., 2007; Munzel et al., 2002).

Another deep-rooted perception regarding the lucigenin superoxide assay is that the readings that are observed are strictly extracellular. This theory has been discredited
in vascular studies in which polar non-membrane permeable antioxidants, such as SOD have been unable to decrease superoxide levels completely, only actually lowering levels by approximately 50% (Dikalov et al., 2007). In contrast, when polar antioxidants which can gain access to the intracellular space, such as tiron, are used to decrease superoxide levels, they can almost completely eliminate the superoxide signal (Munzel et al., 2002). Thus, the chemiluminescence based lucigenin superoxide assay does have limitations with respect to its accuracy and sensitivity, however, virtually all results attained in the literature with lucigenin have been confirmed demonstrating its potential when used properly (Dikalov et al., 2007).

DCF has many similar issues to lucigenin. The main concerns with the DCF technique include: (1) the conversion of oxygen to superoxide through a semiquione produced due to photoreduction, (2) the oxidation of DCF can be self-catalyzed by peroxidases, (3) redox reactions can lead to a DCF-free radical anion which can react with superoxide and inhibit endogenous antioxidants (Dikalov et al., 2007). All of these scenarios lead to an increase in fluorescence that is not related to ROS levels.

X.8.C. In vitro versus in vivo study

When dealing with an in vitro culture situation, the results attained in the in vitro setting may not correctly portray the actual proceedings that occur in vivo. Despite attempts to create an in vivo environment, in vitro conditions are unique to the cell culturing process. One example of these differences lies in the partial pressure of O₂ (pO₂) and O₂ concentration at which the cells are grown. In the incubator environment, cells are exposed to an environment similar to atmospheric conditions. This puts O₂ content of the around 20%, leading to a raised level of O₂ in the medium. Studies suggest
that the pO$_2$ found \textit{in vitro} may be double that it is \textit{in vivo} (Sullivan, Galea, & Latif, 2006). The increased level of O$_2$ \textit{in vitro} versus \textit{in vivo} has ramifications not only due to cell growth as has previously been discussed (Krick et al., 2005; Welsh et al., 1998), but also to the reactions that have been studied throughout this thesis. The production of ROS is dependent on the availability of O$_2$ as a reactant, thus, potentially by exposing cells to a high level of O$_2$, the process of ROS production has been altered. Additionally, adventitial fibroblast characteristics may change in the actual vessel environment when in close proximity to VSMCs, ECs and other cell types found within the vessel. Additionally, by using adventitial fibroblasts from passage 3 for treatment, there is a long period of time between the initially primary culture and the treatment of passage 3 cells. It is unlikely that original cells isolated through primary culture remain in the culture to the passage 3 stage. Thus, the descendents of the cells isolated through primary culture may have some physiological differences versus their precursor cells as they have never experienced an \textit{in vivo} setting. Due to these considerations that must be made with an \textit{in vitro} setting, it will be necessary to test the results found in the \textit{in vitro} setting in an \textit{in vivo} setting in further research.

\textbf{X.9. Conclusion}

Overall, the pathway that this thesis constructed was that ET-1 induces an increase in ROS levels, peaking after 4 hrs of ET-1 treatment in adventitial fibroblasts. This increase in ROS levels is mediated through an ET$_A$ receptor – ERK1/2 MAPK pathway. The increase in ROS production due to ET-1 is primarily due to ROS from NOX2. NOX4 protein levels are elevated due to ET-1 stimulation, however, the time that ROS increases and NOX4 protein increases do not coincide.
Ang II treatment also produces a strong peak in superoxide levels after 1.5 hrs and 6 hrs of stimulation, with the secondary phase being mediated by both an AT₁ receptor and an ETₐ receptor pathway. As the secondary phase is inhibited by both an AT₁ receptor and an ETₐ receptor pathway, my conclusion is that Ang II induces a release of ET-1 via an AT₁ mediated pathway which has previously been demonstrated by us (An et al., 2006; An et al., 2007). This functional ET-1 that is released then causes a subsequent increase in ROS levels via an ETₐ receptor pathway (Figure 15).

X.10. Future Research

Following the results of the present thesis with regards to ET-1 and ROS levels, research in the field of adventitial fibroblasts should focus on the conversion of adventitial fibroblasts to myofibroblasts and the possible involvement of ET-1. The conversion of adventitial fibroblasts to myofibroblasts is known to be detrimental to the functioning of the vessel, particularly in neointimal formation (Dourron et al., 2005; Weaver et al., 2006). With my research demonstrating that ET-1 receptors mediate a significant portion of the elevated superoxide levels due to Ang II stimulation over 6 hrs, it would be interesting to examine what role ET-1 may have in the Ang II induced conversion of adventitial fibroblasts to myofibroblasts (Shen et al., 2006). With the research of Shi-wen et al. (2004) demonstrating that ET-1 has the ability to induce the conversion of pulmonary adventitial fibroblasts to myofibroblasts and the study by Shen et al. (2006) demonstrating that ROS mediated the Ang II-induced conversion, ET-1 may mediate the Ang II induced conversion of adventitial fibroblasts to myofibroblasts. If ET-1 does mediate the Ang II induced conversion of adventitial fibroblasts to
myofibroblasts, it would be interesting to observe what would occur if an ET receptor antagonist or inhibitor was present during the course of neointimal formation.
Figure 15: Illustration of Ang II and ET-1 ROS response mechanisms.

Ang II leads to an increase in ROS levels after 1.5 hours of stimulation through an AT₁ receptor mediated pathway. This increase in ROS leads to ET-1 being released and stimulating cells. ET-1 stimulation increases ROS levels primarily through an ET₄ receptor, ERK1/2, NOX2 pathway. NOX4 protein expression does increase, however, it does not appear to be critical to ET-1 induced ROS production. The maximum increase in ROS due to ET-1 stimulation is observed after 4 hours of ET-1 stimulation.
XII. Reference List


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