eNOS is Present in Vascular Smooth Muscle Cells of Porcine Left Anterior Descending Coronary Arteries

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A Thesis Submitted to the Faculty of Graduate Studies to Fulfill the Requirements for the Degree of Master of Science

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LIST OF ABBREVIATIONS

AB/AM	- antibiotic-antimycotic
ACE	- angiotensin converting enzyme
ACh	- acetylcholine
Ang II	- angiotensin II
BK	- bradykinin
BSA	- bovine serum albumin
DMEM	- Dulbecco's Modified Eagle's Media
DNA	- deoxyribonucleic acid
EC	- endothelial cell
ECM	- extracellular matrix
EDRF	- endothelial derived relaxing factor
eNOS	- endothelial nitric oxide synthase
FBS	- fetal bovine serum
iNOS	- inducible nitric oxide synthase
LADCA	- left anterior descending coronary artery
NO	- nitric oxide
NOS	- nitric oxide synthase
nNOS	- neuronal nitric oxide synthase
OCT	- optimal cutting temperature
PBS	- phosphate-buffered saline
PCA	- porcine coronary artery
PGI_2	- prostaglandin (I_2)/ prostacyclin
PTCA	- percutaneous transluminal coronary angioplasty
RNA	- ribonucleic acid
RO H_20	- reverse osmosis water
RT-PCR	- reverse transcriptase polymerase chain reaction
SM	- smooth muscle
SMC	- smooth muscle cell
SOD	- superoxide dismutase
TBS-T	- Tris-buffered saline with Tween-20®
VSMC	- vascular smooth muscle cell
vWF	- von Willebrand factor

ABSTRACT

Percutaneous transluminal coronary angioplasty is associated with risk of restenosis or re-occlusion of coronary arteries due to loss or damage of endothelial cells upon inflation of the balloon catheter. The resultant loss of endothelial nitric oxide synthase (eNOS) decreases the ability to produce nitric oxide (NO). NO has important vasodilatory, anti-proliferative and anti-migratory effects on vascular smooth muscle cells (VSMCs) and limits the adherence of platelets and leukocytes to the endothelium. However, eNOS was detected in porcine left anterior descending coronary arteries (LADCAs), post-angioplasty. As re-endothelialization cannot occur in our organ culture system, it was suspected that eNOS was present in vascular smooth muscle cells.

Using immunofluorescence, we found that eNOS was expressed in porcine LADCAs post-angioplasty although its expression was variable over 24 hour intervals post injury. Interestingly, eNOS was also detectable in the medial region of both non-injured and balloon-injured vessels as confirmed by gene expression. Statistical analysis of Western blot data showed there was no significant change in eNOS protein levels over time, and between non-injured and balloon-injured samples. However, statistically significant differences were noted for eNOS between VSMCs and native endothelium. Cultured VSMCs explanted from LADCAs were stimulated with either Ang II or serum. Although the results were inconclusive, the addition of Ang II receptor antagonists, losartan and PD123319, did not alter eNOS levels. Unfortunately, eNOS activity could not be determined during the course of these studies.

Our *ex vivo* studies suggest that eNOS is expressed within the medial SMC layer of porcine LADCAs. The time frame for eNOS expression both at the mRNA and protein level remains elusive, however, the effect of injury is apparently an insignificant factor as eNOS is present in non-injured vessels. These findings may be significant since overexpression of eNOS in VSMCs is being considered as one approach to reduce restenosis. With eNOS being present in the native VSMC as demonstrated in these studies, further investigation is needed to determine whether medial eNOS is active and, if it is, to determine whether it has positive or negative effects on vascular function.

1. INTRODUCTION

1.1 Statement of the Problem

In North America, a common cause of mortality and morbidity is heart disease. One of the major causes of heart disease is atherosclerosis which disrupts blood flow to the heart through development of plaque within the coronary artery lumen. One of the methods used to re-establish flow once a vessel becomes occluded is percutaneous transluminal coronary angioplasty (PTCA). This intervention involves inflation of a balloon catheter at the site of occlusion. The major complication associated with this treatment is re-occlusion of the coronary artery, a process also termed restenosis (Hinohara, 2001; Nobuyoshi et al., 1988).

Although atherosclerotic and restenotic lesions result from the migration and proliferation of smooth muscle cells, both restenosis and atherosclerosis are also associated with endothelial cell (EC) disruption (Bailey, 2002; Hong, 2001; George, 1999) and dysfunction (DiCorleto et al., 1996), respectively. EC have a major role in maintaining vascular homeostasis, and this is achieved by the secretion of various paracrine agents. One of these is nitric oxide (NO), a potent vasodilator which is involved in the constant modulation of basal vascular tone, working to balance the effects of other agents within the vasculature. NO also modulates smooth muscle cell migration and proliferation, and prevents platelet aggregation and adherence. The synthesis of NO, a biologically active molecule and free radical gas, is catalyzed by eNOS/NOS III, a constitutive isoform of nitric oxide synthase expressed in the EC layer (Sanders et al., 2000; Lamas et al., 1992).

Clinically, PTCA causes denudation of the vessel, resulting in the loss or reduction of NO synthesis through removal of endothelial cells. As a result, the artery's ability to modulate vascular recoil, intimal hyperplasia and vascular remodeling, three components of restenosis (refer to section 2.2.2) is decreased. For this reason, restenosis (and possibly atherosclerosis) may be coupled to a reduction or modulation of eNOS and/or NO levels. This view is supported by evidence showing that over-expression of eNOS via gene transfer can reduce the incidence of restenosis (Sato et al., 2000; George, 1999; Gurjar et al., 1999; Sharma et al., 1999; Chen et al., 1998a; Janssens et al., 1998; Varenne et al., 1998; Kullo et al., 1997). It is therefore of great interest to understand the conditions that determine physiological expression of eNOS within the medial layer of coronary arteries.

Over the course of this investigation, eNOS expression was detected in coronary arteries placed into organ culture. The novelty of this observation relates to the fact that vessels denuded by balloon-angioplasty were cultured under conditions which do not support re-endothelialization. This finding led to the suspicion that vascular smooth muscle cells (VSMCs) can balance the loss of eNOS in the endothelium by producing eNOS itself. There is precedence for this idea as eNOS expression has been detected in non-vascular SMCs such as rabbit and human gastrointestinal SMCs (Teng et al., 1998) and aortic SMCs of rats with congestive heart failure (CHF) (Comini et al., 1996). In addition, eNOS, which is normally expressed constitutively within the vascular endothelium, can be induced further by exercise, shear stress and cell growth (Sessa et al., 1994; Arnal et al., 1999; Nishida et al., 1992; Arnal et al., 1994). Of note, however, are various reports

which have failed to detect the presence of eNOS in the medial SMC layer of coronary arteries (Brophy et al., 2000; Fukuchi and Giaid, 1999; Picard et al., 1998; Sessa et al., 1992). Nevertheless, studies by Zhang et al. (1995) have demonstrated that transfection of the eNOS promoter into cultured VSMCs leads to detectable activity, albeit these are low compared to ECs. These observations suggest that there is similarity in the basal transcription machinery between endothelial and smooth muscle cells (Zhang et al., 1995), and that eNOS would be active if it were expressed within VSMCs.

1.2 Hypothesis

eNOS is expressed in medial VSMCs of the porcine left anterior descending coronary artery (LADCA).

1.3 Objectives

The focus of this investigation was to determine whether eNOS is present in the media of porcine coronary arteries and whether balloon-angioplasty (injury) plays a role. Furthermore, since injury to the vasculature is known to release various hormonal agents that influence the subsequent repair process, this investigation examined whether one of these agents, angiotensin II (Ang II), and a potent mitogen, serum, influence eNOS expression. Specifically, we decided:

i) To determine whether eNOS protein can be detected in medial smooth muscle in normal and balloon-injured porcine LADCAs and in cultured VSMCs explanted from these vessels.

ii) To examine if the addition of either Ang II or serum alters eNOS expression

levels.

iii) To determine whether eNOS mRNA is synthesized in medial porcine VSMCs

iv) To determine if eNOS expressed by VSMCs is active.

2. LITERATURE REVIEW

2.1 Normal Arterial Physiology

Coronary arteries are made up of three distinct, concentric layers: the tunica intima, media and adventitia. The intima is the first and innermost layer, made up of a single layer of endothelial cells (ECs) and sub-endothelial basement membrane which is separated from the medial layer by an internal elastic lamina. The medial smooth muscle cell layers make up the muscular component of the artery. The outermost adventitial layer, which is separated from the media by an external elastic lamina, consists mainly of fibroblasts, some tissue macrophages and loose connective tissue. The tunica adventitia also includes terminal nerve fibres, which can project onto the outer layers of the tunica media (Gutterman, 1999; Pauly et al., 1997; Hoffman, 1990), and smaller collateral arteries to nourish and maintain the larger vessel.

2.1.1 Endothelial Cell Function

The endothelium of coronary arteries is a continuous monolayer of ECs that acts as an important interface regulating the interaction between blood, blood-borne elements and tissue/cellular components. It functions as a selective, semi-permeable barrier to regulate transcapillary permeability of solutes and water, and protein flux across its membranes (Marsden et al., 1991; Ali and Schumacker, 2002). Its dynamic interaction can be intravascular (to the underlying connective tissue or smooth muscle cells) or extravascular (to the blood-borne agents, e.g. platelets) as its location allows for the endothelial cells to monitor, process, then respond to various stimuli (DiCorleto et al., 1996), both locally and systemically. The endothelial barrier prevents the interaction of

platelet-derived products with the underlying cells or its extracellular membrane (ECM) as their interaction may trigger activation of various enzymes such as collagenases and proteases (Marsden et al., 1991). It can also initiate and modulate an inflammatory response locally at its surface or systemically through the release of various mediators, by up- or down-regulating adhesion molecules on its surface or by trafficking blood-borne cells (Marsden et al., 1991; Ali and Schumacker, 2002).

ECs have both an anti- and prothrombogenic action. By producing agents such as prostacyclin (PGI_2) and nitric oxide (NO), both potent inhibitors of platelet aggregation, ECs help maintain an anti-thrombotic environment (Vane et al., 1990). However, by synthesizing cofactors such as von Willebrand factor (vWF), fibronectin and thrombospondin, the endothelium enhances the adherence of platelets, a prothrombotic function (DiCorleto et al., 1996). Balancing these actions allows for normal blood flow while simultaneously ensuring a coagulation mechanism, for example, to stop hemorrhaging at sites of vascular injury (DiCorleto et al., 1996).

ECs exert a paracrine effect on neighboring vascular smooth muscle cells (VSMCs) by synthesizing and releasing both vasodilators and vasoconstrictors. Direction of the response is normally based on the interaction between the cells and blood-borne components (e.g. hormones, leukocytes, etc). However, the response can also be influenced by mechanical and hemodynamic forces. *In vivo*, such forces include shear stress in the direction of blood flow and cyclical strain (stretch) due to distension in all directions of the vascular wall from transmural pressure (Ballerman et al., 1998). The

mechanisms regulating the endothelial cell response to mechanical forces are not well understood as the "mechanosensor(s)" which activates the signaling pathways has not been identified (Ali and Schumacker, 2002). Furchgott and Zawadzki (1980) discovered that the endothelium was capable of relaxing the underlying VSMCs, and thereby modulating vascular tone, by releasing a substance later determined to be NO. NO, known previously as endothelium-derived relaxing factor (EDRF), and prostacyclin are unstable molecules with endothelium-dependent vasodilating effects, their release being triggered by pulsatile blood flow (Rubanyi et al., 1986; Pohl et al., 1986). Any increase in resistance to flow (increasing flow rate, viscosity or decreasing vessel diameter) will acutely increase the shear stress exerted on the cells, raising tension and causing deformation of the cell, which leads to enhanced transcription of the cyclooxygenase-2 and endothelial nitric oxide synthase (eNOS) genes (Ballerman et al., 1998). The NO synthesized by eNOS has been implicated in the normalization of shear stress and wall strain to regulate SMC remodeling in response to changes in blood flow (Papapetropoulos et al., 1999). Therefore, shear stress does impact normal basal tone.

Other important vasoactive agents are synthesized and/or released from the endothelium. Angiotensin converting enzyme (ACE), which is located on the endothelial cell surface, converts angiotensin I to the potent vasoconstrictor peptide, angiotensin II (Ang II). ACE is also responsible for the inactivation of bradykinin (BK), a vasodilator which acts by increasing prostaglandin E_2 and prostacyclin (Vane et al., 1990; Hoffman, 1990). Endothelin-1 is a peptide synthesized and released only by ECs (Yanagisawa et al., 1988) and has vasoconstrictor effects with 10 times the potency of Ang II (Loutzenhiser et al.,

1990; Gryglewski et al., 1988). ECs can also produce various cytokines (e.g. IL-1β, MCP-1), growth factors and inhibitors (e.g. bFGF, PDGF) to modulate locally the action of the neighboring cells, blood components (paracrine) or itself (autocrine) (DiCorleto et al., 1996).

Therefore, the endothelium has a critical role in regulating functions such as permeability, thrombogenicity and basal vascular smooth muscle tone. However, in pathological situations such as those found with vascular disease or endothelial dysfunction, the control and thus the balance between the EC functions are lost. For example, in the study by Furchgott and Zawatzki (1980), the agonist acetylcholine (ACh) induced vasodilation in intact rabbit arteries; however, in vessels where the endothelium was removed, stimulation with ACh had the opposite vasoconstrictive effect. Ludmer et al. (1986) also found the same vasoconstricting effects in response to ACh in atherosclerotic vessels associated with the dysfunctional endothelium.

2.1.2 Vascular Smooth Muscle Cells

Smooth muscle cells are the major cell type of the medial layer and are normally arranged circumferentially within the vessel wall. They are highly specialized cells whose main function is contraction (Owens, 1995). In addition, VSMCs help to regulate vascular tone but can be involved in growth, remodeling and repair in response to injury (Pauly et al., 1997). VSMCs contain molecules such as SM myosin, SM actin, caldesmon and calponin that are necessary for and contribute to the contraction of vessels in response to fluctuations in intracellular calcium levels (Sartore et al., 1999; Owens,

1995). Similarly, molecules such as calmodulin, myosin light chain kinase (MLCK) and type I myosin phosphatase (MLCP) also play a role in the initiation of smooth muscle contraction (Pfitzer, 2001).

2.1.2.1 Characteristics of Differentiated VSMC

Mature, differentiated VSMCs found in the adult arterial wall are contractile, spindle-shaped cells which are quiescent or proliferate at very low rates. These cells are not terminally differentiated as they retain the ability to undergo rapid and reversible changes in their phenotype, a process known as phenotypic modulation (discussed in 2.1.2.2). Environmental cues are normally required to maintain the differentiated state. VSMCs have been found to exhibit different phenotypes in response to different stimuli. Identification of cell phenotype depends on protein expression as well as the morphology, function and anatomical location of the VSMC (Owens, 1996a). The cytoplasm of differentiated VSMCs contains abundant myofilaments with few synthetic organelles. The predominant intracellular protein is SM α-actin (Thyberg et al., 1983), one of the last markers of differentiated SMC to be lost when cells undergo phenotypic modulation to a synthetic, proliferative state (Owens, 1996a). Mature VSMCs from human aorta contain high amounts of the contractile proteins α- SM actin, SM myosin heavy chains, metavinculin and caldesmon. Other recently reported markers of differentiated VSMCs, shown by the presence of genes, are aortic preferentially expressed gene-1 (APEG-1), the nuclear protein smooth muscle LIM protein (SmLim) both found in rat aortic SMCs, and aortic carboxypeptidase-like protein (ACLP) studied in mouse aortic SMCs (Chen et al., 2001; Hsieh et al., 1999; Jain et al., 1996; Layne et al., 2002; Layne et al., 1998). In

contrast, VSMCs from atherosclerotic fibrous plaques contain decreased amounts of both SM actin and myosin heavy chain. Instead, cytokeratin 8 (an intermediate filament) and non-muscle variants of the aforementioned proteins (actin, myosin, vinculin and caldesmon) expressed normally in developing VSMCs are present, suggesting that VSMCs forming intimal lesions revert to a more fetal or immature phenotype (Glukhova et al., 1991; Owens, 1996a).

2.1.2.2 Phenotypic Modulation of VSMCs

Phenotypic modulation refers to alterations in the differentiated state of VSMCs. For example, placing adult SMCs into culture induces a transformation from a contractile to a synthetic phenotype (Rybalkin et al., 2002; Chamley-Campbell et al., 1979). Synthetic, dedifferentiated VSMCs are markedly different from their mature contractile counterparts. Synthetic VSMCs become fibroblast-like in appearance rather than the spindle shape the cells have in the contractile state. They exhibit a high proliferative capacity and synthesize increased amounts of ECM components such as fibronectin and collagen types I and III (Pauly et al., 1997). There is an increase in the RNA-to-DNA ratio, protein-to-DNA content and cell surface area (Powell et al., 1996). There is an increase in glycogen granules, Golgi apparatus, rough endoplasmic reticulum (RER) and the numbers of ribosomes, with a decrease in the numbers of myofilaments (Thyberg et al., 1983; Pauly et al., 1997) which therefore decreases the contractility of VSMCs as they enter the synthetic state. During the development of intimal hyperplasia, the plasticity or ability to manifest a phenotypic switch to the synthetic form precedes migration, proliferation and increased matrix synthesis (Powell et al., 1996; Owens,

1996b). Furthermore, the ability of synthetic VSMCs to proliferate is thought to play a major role in restenosis, post-angioplasty.

2.1.2.3 Regulation of VSMCs

The phenotypic plasticity of VSMCs has made the effort to understand and determine the mechanisms involved in regulating VSMC differentiation, challenging (Halayko and Solway, 2001). There has been no "master" regulatory gene identified in smooth muscle similar to ones found in skeletal muscle (Owens, 1996a). While the complex regulation of VSMCs is not well understood, it is thought that maintenance of the "differentiated" state relies on strict, continuous regulation from various cues (Blau, 1991). Regulation of specific gene expression (e.g. contractile proteins) may be one aspect that controls which phenotype is expressed. In the progression from fetal to adult VSMCs, there is down-regulation of non-muscle protein expression and up-regulation of SM-specific differentiation markers such as SM myosin heavy chains (SM1 is expressed early in development and SM2 is expressed post-natally), SM α-actin, SM22, calponin and h-caldesmon (Sartore et al., 1999; Owens, 1995).

Several external factors may affect the rate and the degree of phenotypic modulation. Placing cells into culture is itself a stimulus as the change in environment causes mature VSMCs *in vivo* to express a more synthetic phenotype (Owens, 1996a). Acquiring cell confluence in culture, where there is a greater degree of cell-cell interaction, is reflected in increases in the resistance of VSMCs to change. Likewise, SMC dediffentiation does not occur in serum-free media (Chamley-Campbell et al., 1979). Mechanical forces such

as that applied by Kanda and Matsuda (1994) found that mechanical stretch of cultured VSMCs within a three-dimensional type I collagen matrix increased contractile myofilaments and dense bodies in the cells. Birukov et al. (1995) also found that cyclic stretch of cultured SMC can both enhance growth or possibly differentiation depending on whether the media contained high or low level serum, respectively. The ECM can influence the ability of VSMCs to migrate, proliferate and differentiate (Owens, 1996a). Thus, the type of matrix on which the cells are plated may alter SMC morphology, as fibronectin (Hedin et al., 1989) and collagen (type I) promote the synthetic form, while elastin and laminin inhibit the change (Powell et al., 1996; Yamamoto et al., 1993).

Phenotypic modulation of VSMCs can occur in response to substances released from nearby ECs (discussed in 2.1.2.4), and from parenchymal cells and nerve endings that stimulate the outer medial layers from the adventitial aspect of the vessel (Hoffman, 1990). For example, agents such as Ang II and serotonin augment vasoconstriction by stimulating the formation, release of or reducing the re-uptake of the neurotransmitter, norepinephrine (NE) (Ginsburg et al., 1984) from pre-synaptic junctions on the outer aspect of the tunica media. Similarly, vasodilation can occur by stimulation of cholinergic receptors or by prostaglandins (I or E series) both of which inhibit the release of NE, by release of NO or atrial natriopeptide (Hoffman, 1990). These substances act on VSMC receptors and ion channels to modulate contraction by affecting electrical activity or increasing cell sensitivity to these hormones, neurotransmitters and agonists/antagonists (Owens, 1996a). The types of receptors (e.g. angiotension (AT_1 and AT_2) and adrenergic (α_1 and β)) (Viswanathan et al., 1991; Schell et al., 1992; Shaul et

al., 1990) and ion channels vary among different vascular beds and appear during different maturation stages of the SMC (Duckles and Banner, 1984).

2.1.2.4 Endothelial/Smooth Muscle Cell Interaction

Interaction of the EC and SMC cell types *in vitro* results in a contractile, non-synthetic smooth muscle cell phenotype similar to that found in the normal vessel. This was demonstrated by experiments involving both pure SMCs and mixed EC-SMC cultures (Powell et al., 1996). SMCs co-cultured with ECs display the differentiated, contractile phenotype and evenly distributed growth without the "hill and valley" configuration, characteristic of pure SMC cultures. The "hill and valley" description refers to a pattern of growth where areas of densely populated SMCs form mounds while the adjacent, less populated areas are much thinner. SMCs cultured alone tend to enter the synthetic, dedifferentiated contractile phenotype (Powell et al., 1996). The cause of SMC modulation may be related to growth factors, ECM and direct transmembrane signal transduction which may be initiated from both the VSMC and EC (Powell et al., 1996). Recall, hemodynamic/mechanical forces such as shear stress act upon ECs to influence the underlying VSMC, *in vivo*. The pulsatile flow of blood is one environmental cue that allows for continual regulation of vascular tone. This stimulus maintains the differentiated, contractile phenotype necessary for the cell to ultimately sustain its ability to contract and relax. In atherosclerotic vessels, the presence of a plaque/lesion may alter blood flow and thus disrupt the regulatory effect normally found in healthy vessels. The link between SMC phenotype and growth modulation by ECs is suspected to play a role in the pathological conditions of intimal hyperplasia and restenosis (Powell et al., 1996).

Regulation of tone and therefore resistance to flow have implications on systemic blood pressure and perfusion of blood to other organs. Any dysfunction in the control of vessels may further contribute to the progression of these and other pathological conditions.

2.1.2.4.1 Effects of Angiotensin II

Ang II is a hormone produced both locally and systemically by the renin-angiotensin system. Renin, a circulating or local enzyme, converts angiotensinogen into angiotensin I within plasma. ACE, found on the luminal surfaces of ECs and in VSMCs, catalyzes the cleavage of Ang I to the active octapeptide, Ang II (Duff et al., 1995; Berk et al., 1996). The effects of Ang II are mediated mainly through two receptor types, the AT_1 and AT_2 receptors, whose effects can be inhibited by the specific antagonists, losartan (DuP 753) and PD123319, respectively (Seyedi et al., 1995; Millatt et al., 1999). These receptors belong to the 7-transmembrane domain family suggesting their actions are mediated through G protein-coupled signal transduction pathways (Cascieri et al., 1996).

Ang II is a potent vasoconstrictor that acts directly on VSMCs via AT_1 receptors to influence blood pressure (Peach, 1977). There is evidence to suggest that Ang II may also have vasodilatory effects, since Ang II can effect nitric oxide (NO) release from cultured rat aortic ECs (Pueyo et al., 1998). A number of studies have implied that Ang II stimulates the release of NO either by increasing the levels of intracellular calcium or through local kinin formation (Pueyo et al., 1998; Seyedi et al., 1995). Seyedi and co-workers (1995) have found that Ang I, Ang II and its fragments (Ang III and IV) increase

nitrite release, the breakdown product of NO, in canine coronary arteries. The angiotensin-induced increase in NO was prevented by NOS inhibitors, bradykinin (BK) B_2 receptor antagonists and protease inhibitors which block local kinin formation (Seyedi et al., 1995), clearly suggesting that NO release was mediated through kinin formation or stimulation of its receptors, although the exact mechanism is unknown. It was also found by Pueyo et al. (1998) that although Ang II stimulates NO, it simultaneously produces peroxynitrite, a powerful oxidant formed when NO combines with $O_2^{\bullet-}$ which is a superoxide anion known to damage cell membranes (Goldstein and Czapski, 2000; Pryor and Squadrito, 1995). The effects of Ang II depend with which cell type (EC or SMC) Ang II first interacts (Pueyo et al., 1998). There also appears to be some tissue specificity as Ang II stimulated an increase in eNOS mRNA, protein and NO production in cultured bovine pulmonary ECs, whereas there was no effect on cultured bovine coronary artery ECs (Olson et al., 1997).

Ang II also stimulates VSMC growth. Infusion of Ang II induces vascular wall hypertrophy and proliferation of SMCs *in vivo* (Daemen et al., 1991), similarly the action of Ang II on cultured VSMCs cause an increase in the mRNA and protein synthesis (Owens, 1989). Similarly, Geisterfer et al. (1988) found Ang II induced hypertrophy but not hyperplasia in cultured rat aortic VSMCs. It has also been found that Ang II infusion *in vivo* stimulates neointimal proliferation in balloon-injured vessels (Daemen et al., 1991). Blocking Ang II effects with an ACE inhibitor or Ang II receptor antagonist prevented neointimal growth and/or proliferation in a rat carotid injury model (Powell et al., 1989). This may be partly through the effects of BK, normally inactivated by ACE

(Linz et al., 1999; Hoffman, 1990), as BK is known to stimulate the release of NO, which reduces neointimal proliferation. On a similar note, enhanced expression and activity of vascular ACE with its associated increase in Ang II formation or breakdown of BK, has been associated with endothelial vasomotor dysfunction through impairment of the available endothelium-derived NO (Goetz and Holtz, 1999a). This suggests a role for Ang II in the physiology and pathophysiology of the vasculature and the related diseases hypertension, atherosclerosis and restenosis post-angioplasty (Duff et al., 1995; Berk et al., 1996; Goetz and Holtz, 1999a).

2.1.2.4.2 Effects of Nitric Oxide on VSMCs

NO is synthesized by a family of enzymes termed nitric oxide synthase (NOS) that consists of three isoforms: nNOS/NOS I, iNOS/NOS II and eNOS/NOS III. All three isoforms of NOS have been detected in vascular tissues (Wilcox et al., 1997). In the presence of various cofactors, calmodulin and increased levels of intracellular calcium, the NOS isoform found in ECs (eNOS) is constitutively active. Endothelial NO subsequently diffuses to the VSMCs where a soluble guanylyl cyclase (GC) is activated (Sanders et al., 2000). Similarly, inducible NOS (iNOS) expression can be induced in VSMCs by cytokines and other inflammatory mediators (Fleming et al., 1991; Koide et al., 1994) to produce large amounts of NO directly within the SMC. Activation of GC leads to elevated levels of the secondary messenger cGMP, which is believed to mediate the biological actions of NO by activating a cGMP-dependent protein kinase (Millatt et al., 1999). NO inhibits SMC proliferation and migration as well as platelet and leukocyte aggregation and adhesion, while stimulating vasodilation. Although Ang II can affect the

release of NO, NO is also thought to down-regulate Ang II receptors in a tissue-specific manner (Millatt et al., 1999). An inverse correlation has also been found between ACE expression and NO, where ACE inhibition not only stimulated NO synthesis but also induced expression of the eNOS gene (Linz et al., 1999).

2.1.3 Adventitia

In the past, the adventitia was considered to be only a support structure for the vessel, and whose main function was to nourish the underlying medial layer (Sartore et al., 2001). However, it has been recognized that the adventitia may have an active role in regulating vascular function. In the normal artery, non-muscle cells such as fibroblasts, the vasa vasorum and nerve terminals are found within the adventitia.

Autonomic nerve fibers (e.g. parasympathetic) enter from the adventitial aspect but do not penetrate much beyond the border between the adventitia and the media. The nerve fibers, which may be noradrenergic, cholinergic or nonadrenergic-noncholinergic (NANC), send extensions that form varicosities on the outer surfaces of the media from where the neurotransmitters diffuse to the adjacent muscle cells (Kurihara et al., 1995; Hoffman, 1990). NO also acts as a neurotransmitter since it is released from NANC-I nerve endings (Ursell and Mayes, 1993; Ignarro, 1996) in cerebral arteries and stimulates vasodilation.

Similar to medial SMCs, fibroblasts can proliferate, migrate and undergo differentiation to form myofibroblasts, which are contractile and can synthesize ECM proteins (Zalewski

and Shi, 1997). The adventitial cells are thought to play a role in the initiation and progression of atherosclerosis. In one study, Barker and co-workers found that removal of the adventitia from the large arteries of rabbits caused intimal and medial hyperplasia similar to early atherosclerotic lesions (Barker et al., 1994). Furthermore, Scott et al. (1996) reported that bromodeoxyuridine (BrdU) labeling of proliferating cells in the vascular wall was highest in the adventitia 3 days after balloon-injury, whereas proliferation was highest in the neointima 7 days post-injury. Based on these results, it was proposed that the adventitia may be a source of neointimal cells, in addition to the medial cell layer.

2.2 Vascular Disease

Cardiovascular disease is the leading cause of morbidity and mortality in North America. Diseases such as atherosclerosis and restenosis, where obstruction of blood flow causes ischemia of cardiac tissues triggering angina, predisposes coronary vasospasm and thrombus formation (Fuster et al., 1992) and may lead to myocardial infarction. Numerous risk factors can increase the chances of developing cardiovascular disease and although some factors are modifiable by individual behaviour (smoking, hypercholesterolemia, diet, hypertension, diabetes mellitus, sedentary lifestyle), others are not (age, gender, family history) (Kannel et al., 1976a; Kannel, 1976b). Endothelial dysfunction and the proliferation and migration of VSMCs are key events in the development of atherosclerosis, and the incidence of such events is increased in individuals with hypertension and diabetes (Ross, 1993 and Suzuki et al., 2001). Therefore, efforts to understand and control VSMCs are ongoing.

2.2.1 Atherosclerosis

Clinically apparent atherosclerosis takes decades to develop (Adams et al., 2000). It begins with an early fatty streak and progresses over time into an atheroma or plaque that is surrounded by a protective fibrous cap comprised of VSMCs and ECM. The cap contains a lipid core, over which lies the endothelium. Consequently, over time, EC structure and function are altered by these pathological changes. Increased plasma membrane permeability, modification of plasma proteins, oxidation of LDL, increased adhesiveness for leukocytes and imbalances between vasoactive agents, anti/ prothrombic substances and growth factors/inhibitors may all result from endothelial dysfunction (DiCorleto et al., 1996). Long-term cellular dysfunction may lead to chronic reduction in lumen diameter, destabilization of the plaque, and the development of thrombi (Rosenfeld, 2000).

It was previously believed that the initiating factor for atherosclerosis was an undefined injury of the endothelium allowing exposure of the highly thrombogenic underlying basement membrane (Ross et al., 1976). However, it has been found that denudation does not occur in the early stages of atherogenesis but is found in areas with advanced plaque formation (DiCorleto et al., 1996). Possible insults causing endothelial injury may be altered shear stress, homocysteine, inflammatory cytokines, free radicals and other chemicals (Adams et al., 2000). Disturbed blood flow, whether turbulent and/or slow, can have pathological consequences for the endothelium due to alteration of normal shear stress forces. Studies have found that areas of abnormal hemodynamics lead to

vessels being at higher risk for development of atherosclerotic plaque (Silacci et al., 2000; Nerem, 1993; Comini et al., 1996). The development and enlargement of arterial plaque further disturbs blood flow (Adams et al., 2000) and reinforces disease progression.

Studies in both humans and animals have found an inverse relationship between atherosclerotic plaque development and vasorelaxation (DiCorleto et al., 1996). Furchgott et al. (1980) discovered that removal of the endothelial lining impaired the ability of vessels to relax in response to acetylcholine. Similarly, reduction or loss of NO synthesis and the resulting impairment in endothelium-dependent vasodilation can lead to atherosclerosis (Marsden et al., 1991; Wilcox et al., 1997).

2.2.2 Restenosis

Management of coronary artery diseases such as atherosclerosis typically involves a method of revascularization like percutaneous transluminal coronary angioplasty (PTCA) or coronary artery by-pass grafts which help re-establish blood flow distally. The use of percutaneous coronary interventions has increased to as many as 800,000 procedures performed annually in the US (Topol, 1998). The major post-procedural complication involves re-occlusion or restenosis of the vessel. Restenosis occurs in 30-50 % of all cases within 6 months of the PTCA procedure (Hinohara, 2001; Nobuyoshi et al., 1988).

There are three overlapping but identifiable stages of restenosis following balloon-angioplasty (Bailey, 2002; Hong, 2001; George, 1999). 1) Immediately post-angioplasty,

there is an elastic recoil following removal of the endothelium at the site of balloon-injury and accumulation of platelets, thrombus and inflammatory cells. 2) This is followed by (neo)intimal hyperplasia or cellular proliferation and migration and production of excess ECM by VSMCs into the injured area beginning after 2-3 days (Adams et al., 2000; Madri et al., 1991). 3) Even months post-intervention/injury, there is non-cellular growth of the neointima due to increased ECM formation and vascular remodeling of the artery (Adams et al., 2000).

Removal of the endothelium, either through injury or vessel harvesting, is believed to be a key issue in restenosis. Impaired vasomotor tone, changes to thrombogenicity and abnormal shear stress responses persist in some cases post-injury, even after endothelial regeneration (Angelini et al., 1992; Shimokawa et al., 1987). Shimokawa et al. (1987) found re-endothelialization can occur within 8 days after balloon denudation of porcine LADCAs, although the regenerated endothelium appeared morphologically different compared to native cells. This denuded state contributes to VSMC proliferation and vascular remodeling. Inflammation and matrix formation may also contribute to restenosis (Bailey, 2002). Thus, disruption of the endothelial layer during revascularization procedures can pre-dispose the vessel to an increase in (neo)intimal thickness that may elevate the risk for thrombosis.

As previously discussed, VSMCs within these neointimal lesions show an altered, more synthetic phenotype (Pauly et al., 1994), unlike the differentiated form found normally within the media. This modulation of phenotype may be due to the lack of balancing

factors emitted from the endothelium, such as NO, or that are released from platelets. Direct injury to the medial VSMC may also cause oxidative stress which has been shown to activate pathways leading to altered gene expression (e.g. such as those regulating ECM synthesis (Kibbe et al., 1999)) or stimulate a response by VSMCs to produce factors such as bFGF and Ang II (Adams et al., 2000; Schwartz et al., 1996). Ang II can have effects on both ECs and SMCs as described in sections (2.1.2.4.1) and (2.3.5). Therefore, ECs, SMCs and their interactions are important aspects for study, and may be potential targets for therapeutic intervention to reduce and/or prevent restenosis.

2.3 Nitric Oxide Synthase

The nitric oxide synthase (NOS) family of enzymes are cytochrome P_{450}-like hemoproteins (White and Marletta, 1992) which produce the potent free-radical gas nitric oxide (NO). The reaction catalyzed by NOS is shown in Figure 1. NO, originally referred to as endothelium-derived relaxing factor (EDRF), has many biological effects on mammalian physiology. NO can act as a neurotransmitter involved in long-term potentiation and depression, as a regulator of vascular tone and cardiomyocyte contractility and even as a cytotoxic agent in immune or inflammatory reactions. These actions have been reviewed by Ignarro et al. (1996), Feldman et al. (1993) and Nathan et al. (1992). Of note, in 1998, Drs Robert F. Furchgott, Louis J. Ignarro and Ferid Murad won the Noble Prize in physiology or medicine "for their discoveries concerning NO as a signaling molecule in the cardiovascular system".

Figure 1: NO Synthesis Catalyzed by Dimerization of eNOS Monomers

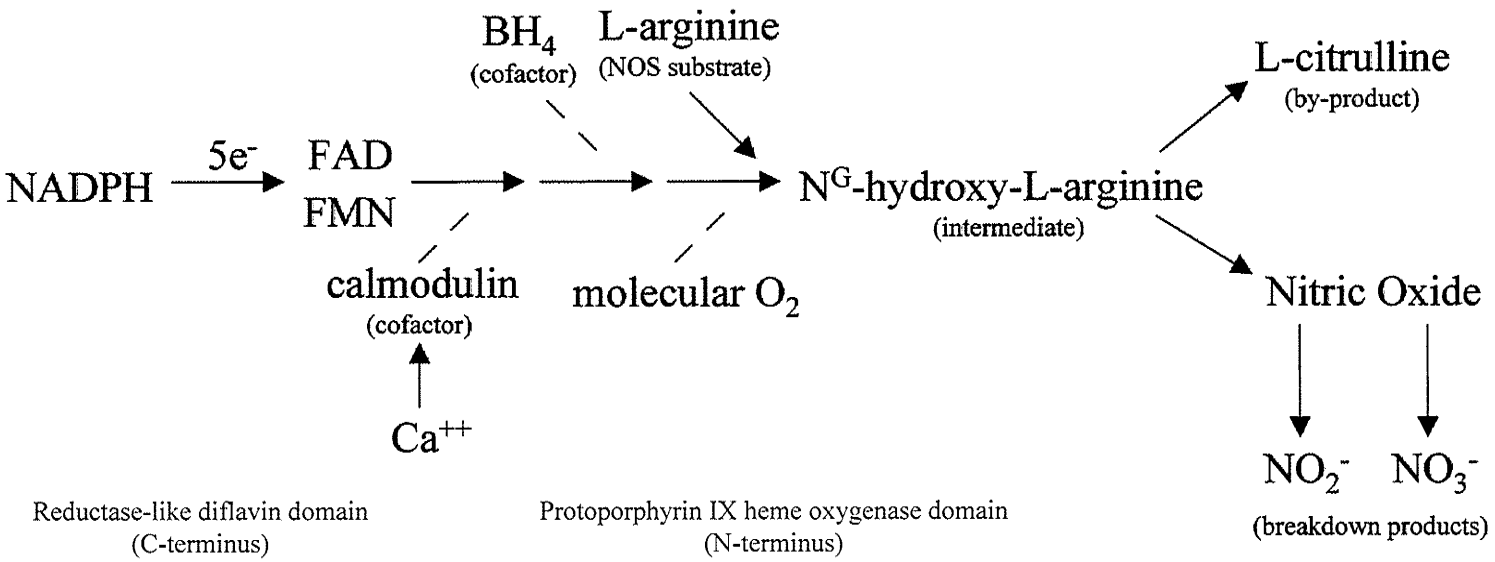


Diagram based on descriptions of activity within each NOS oxygenase monomer.
References: Panda et al., 2002; Andrew and Mayer, 1999; Stuehr, 1997; Griffith and Stuehr, 1995; Marletta, 1993; Abu-Soud and Stuehr, 1993; White and Marletta, 1992, Hevel et al., 1991

There are three major isoforms of NOS: i) neuronal or nNOS, ii) endothelial or eNOS, and iii) inducible or iNOS. Although the names derive from the cell source from which they were initially characterized, all three NOS isoforms are found in numerous cell types and tissues. Neuronal NOS is present in gastrointestinal myenteric neurons and smooth muscle cells (SMCs), developing and adult rat skeletal muscle cells, VSMCs and even in advanced atherosclerotic ECs (Chakder et al., 1997; Tidball et al., 1998; Brophy et al., 2000; Wilcox et al., 1997). Inducible NOS was initially found in macrophages but can be expressed in almost all nucleated cell types including VSMCs, rat thoracic/rabbit aortic SMCs, neonatal trabecular osteoblasts and osteoclasts (Wilcox et al., 1997; Koide et al., 1994; Busse et al., 1990; Fleming et al., 1991; Hukkanen et al., 1999). Endothelial NOS can be expressed in rat aortic SMCs during congestive heart failure (CHF), human and rabbit gastrointestinal smooth muscle cells and rat skeletal bone (Comini et al., 1996; Teng et al., 1998; Hukkanen et al., 1999). Cells can often express more than one NOS isoform. For example, it has been found that cardiac myocytes and human platelets as well as vascular endothelium can co-express eNOS and iNOS (Ikeda and Shimada, 1997; Mehta et al., 1995; Zhang et al., 2000; Kroll and Waltenberger, 1998; Zhang et al., 1997). Similarly, there can be multiple NOS expressed within the same structure. In the hippocampus, pyramidal (CA1) cells express eNOS whereas nNOS is restricted to the neurons (Dinerman et al., 1994). Likewise in atherosclerotic lesions, all NOS isoforms may be present (Wilcox et al., 1997). Therefore, the original nomenclature for NOS can be misleading. NOS isoforms have therefore been given new designations by the Human Genome Nomenclature Committee in the order of their isolation or molecular characterization:

NOS I (nNOS, (Bredt et al., 1991))

NOS II (iNOS, (Xie et al., 1992))

NOS III (eNOS, (Lamas et al., 1992))

2.3.1 General NOS Protein Structure and Catalytic Mechanism

Nitric oxide synthase isoforms I, II and III possess a reductase domain that is similar to cytochrome P_{450} reductase (Wang et al., 1995b). However, NOS isoforms differ from cytochrome P_{450} reductase by being "the first catalytically self-sufficient P_{450} enzyme with both a reductase and an oxygenase domain" on the same polypeptide strand (White and Marletta, 1992). All NOS isoforms have catalytic sites (Mayer and Hemmens, 1997; Stuehr, 1997) consisting of two functional domains. The carboxy-terminal reductase domain includes the FAD, FMN and NADPH binding sites, while the amino-terminal oxygenase domain contains a bound protoporphyrin IX heme, a binding site for (6R) 5,6,7,8-tetrahydrobiopterin (BH_4) and L-arginine, and a site for binding calmodulin (Hevel et al., 1991; Mayer, 1998; Andrew and Mayer, 1999; White and Marletta, 1992).

Neuronal NOS and endothelial NOS are constitutively expressed isoforms producing low basal levels of NO in the absence of any particular stimulus. They are Ca^{++} and calmodulin-dependent enzymes, requiring both cofactors for activity and/or further activation by a stimulus to produce increased levels of NO. In contrast, inducible NOS can be stimulated by either cytokines or bacterial endotoxins to produce large and potentially toxic amounts of NO. Of the three NOS isoforms, iNOS can synthesize the highest amount of NO once activated. Inducible NOS is Ca^{++}-independent, although it

requires the presence of calmodulin for NO production (Mayer, 1998). Despite the use of the terms constitutive and inducible, it is now known that expression of both eNOS and nNOS can be induced (see section 2.3.3) and, similarly, iNOS may act constitutively under different physiological conditions (Michel, 1999).

All NOS isoforms are functional dimers. For constitutive NOS (eNOS, nNOS), an increase in intracellular Ca^{++} concentration or possibly the sensitivity to normal Ca^{++} levels (Michell et al., 2002) allows the binding of Ca^{++}-calmodulin to the protein, which promotes the interaction of their monomeric oxygenase domains. Calmodulin binding is also essential for NADPH-derived electron flow from the reductase to the heme group of the oxygenase domain (Mayer, 1998; Abu-Soud and Stuehr, 1993). Typically, eNOS has lower activity than nNOS or iNOS because of the "lower ability for its flavoprotein reductase domain to transfer electrons to the catalytic heme domain" (Nishida et al., 1998). However, it is suggested that the strength of dimer interaction is strongest in eNOS, followed by nNOS and iNOS (Panda et al., 2002). Dimerization of the eNOS oxygenase domain appears not to be dependent on L-arginine or BH_4 (Hellermann et al., 1997; Rodriguez-Crespo et al., 1996), however, heme is thought to play an essential role (Andrew and Mayer, 1999). It has been reported that heterodimeric complexes can form between full-length and truncated (mutated) eNOS (Lee et al., 1995). Upon dimer formation, it is thought that electrons donated from NADPH are "shuttled" directly from the flavins in the carboxy-terminus to the heme moiety in the amino-terminus. This allows formation of a perferryl Fe-heme complex ($Fe(O)^{3+}$), which could catalyze the formation of the intermediate, N^{ω}-hydroxy-L-arginine (White and Marletta, 1992).

Additional requirements are molecular oxygen, L-arginine (the NOS substrate) and BH_4, an essential cofactor thought to provide stabilization of the monomers for electron transfer from the flavins to heme (Marletta, 1993; Mayer, 1998). NOS activation eventually results in the 5-electron oxidation of the guanidino nitrogen of L-arginine to generate NO and the by-product, L-citrulline (Abu-Soud and Stuehr, 1993).

2.3.2 NOS Gene Structure

Genes encoding NOS enzymes have been cloned and functionally expressed in various cell types (Nakane et al., 1993; Geller et al., 1993; Marsden et al., 1992; Janssens et al., 1992). Studies on genomic organization have revealed a high degree of similarity among all NOS genes which may reflect past gene duplication and chromosome rearrangement events that could explain the structural similarity and chromosomal diversity of the NOS isoforms (Wang et al., 1995a). The human nNOS/NOS I gene consists of 29 exons found on chromosome 12 (12q24.2). The full-length open reading frame (ORF) is 4302 base-pairs (bp) encoding a 1434 amino acid (a.a.) protein weighing approximately 160 kDa. Its translational initiation site is within exon 2, whereas its termination site is found within exon 29 (Hall et al., 1994; Wang et al., 1995a). The human iNOS/NOS II gene is found on chromosome 17 (17 q11.2-12) and consists of 26 exons. The ORF (3459 bp) encodes a 1153 a.a. protein with a mass of approximately 130 kDa. Its translational initiation site is within exon 2 whereas its termination site is found within exon 26 (Chartrain et al., 1994). The human eNOS/NOS III gene is located on chromosome 7 (7q35-36) where it contains 26 exons, with an ORF of 3609 bp encoding a 1203 a.a. protein of 135-140 kDa. Its translational initiation site is found within exon 1 and its

termination site in exon 26 (Marsden et al., 1992). By comparing the amino acid sequence of available eNOS sequences (various mammals and *Xenopus*) or of the three human NOS isoforms, there is evidence of NOS sequence conservation throughout evolution. Comparisons between human and bovine eNOS reveal a 94% amino acid sequence identity, whereas there is a 50% and 60% identity with murine macrophage-derived iNOS and rat neuronal NOS, respectively (Marsden et al., 1992). Overall amino acid sequence identity between the three NOS isoforms is 55% with strong sequence conservation in regions associated with catalysis (Michel et al., 1999). The amino-terminal (oxygenase) domain may confer the uniqueness of each NOS isoform, whereas the carboxy-terminal (reductase) domains are highly homologous (Nishida et al., 1992).

2.3.3 eNOS Gene and Protein Regulation

Endothelial NOS/NOS III, normally expressed constitutively within the endothelium, is regulated at both the gene and protein level. The eNOS gene can be induced or reduced by various stimuli. Expression of eNOS is altered in ECs exposed to various mechanical forces via both transcriptional and post-transcriptional mechanisms (Ziegler et al., 1998). More specifically, increased eNOS mRNA and protein were found after ECs were exposed to exercise and shear stress (Sessa et al., 1994; Nishida et al., 1992). Hypoxia can inhibit eNOS expression at the transcriptional and post-transcriptional level. Changes in oxygen concentration from 95% to 3% cause a progressive decrease in eNOS mRNA by shortening the half-life of eNOS mRNA from 46 to 24 hours (by decreasing the stability of the transcript) and repressing eNOS gene transcription by 20-fold (Liao et al., 1995). Similar studies show that exposure to low pO_2 for 24 hours causes a 40-60%

reduction in steady state mRNA eNOS levels due to decreased transcription of the eNOS gene and decreased message stability (McQuillan et al., 1994). Inflammatory cytokines, IFNγ + IL-1β or IFNγ +TNF increased eNOS activity by elevating endogenous BH_4 levels, although the steady state levels of eNOS mRNA also decrease by 94% (Rosenkranz-Weiss et al., 1994). Interestingly, TNFα destabilized eNOS mRNA by decreasing its half-life from 48 to 3 hours in human umbilical vein endothelial cells, a process dependent on new protein synthesis (Yoshizumi et al., 1993). Angiotensin II has also been reported to increase eNOS mRNA and protein in bovine pulmonary artery but not coronary artery endothelial cells, whereas eNOS expression with or without Ang II stimulation was not detectable in pulmonary artery smooth muscle cells (Olson et al., 1997). Therefore, there appears to be mechanisms regulating eNOS activity, protein and mRNA stability, synthesis and responsiveness to stimuli (O_2, Ang II, TNF α), some of which are cell type specific (EC, VSMCs).

Ca^{++}-calmodulin-induced eNOS activation can be triggered by physical stimuli (Sessa et al., 1994) or hormones, much like eNOS expression. However, it is important to note that there is tissue-specificity not only for gene expression but also for eNOS activation. For example, rapid eNOS activation by estrogen was seen in ovine fetal pulmonary artery endothelial cells and human bronchiolar airway epithelial cells, but not in cultured human uterine endothelial and myometrial SMCs (Pace et al., 1999; Kirsch et al., 1999; Tschugguel et al., 1997). Differences in activity have also been reported between native, freshly isolated cells versus their cultured counterparts (Hecker et al., 1994; Busse et al., 1990). Hypoxia-induced inhibition of eNOS expression in human cultured (umbilical

vein) ECs occurs by reducing transcription and mRNA stability (McQuillan et al., 1994), as previously mentioned. In contrast, hypoxia caused eNOS activation and NO production in cultured porcine coronary resistance arteriolar endothelial cells (Xu et al., 1995). The pH of the environment surrounding eNOS also plays an important role in controlling NOS activity as 1 pH unit above or below the pH 7.6 optimum abrogates eNOS activity. This may be associated with its response to hypoxic conditions. The pH optimum for iNOS is pH 7.8-8.0 whereas for nNOS, the optimum is pH 6.7 (Hecker et al., 1994; Stuehr et al., 1992).

The active form of eNOS, like all NOS isoforms, is a homodimer that forms between two oxygenase monomers upon binding of Ca^{++}-calmodulin (Hellerman et al., 1997). However, the eNOS protein as well as nNOS (but not iNOS), is reported to contain a 52-55 a.a. autoinhibitory loop within the FMN binding reductase domain that is thought to play a role in the transfer of electrons to the oxygenase domain. This insert inhibits the activation of eNOS and is thought to interfere with Ca^{++}-dependent binding of calmodulin (Salerno et al., 1997), contributing to the low overall activity of eNOS (Nishida et al., 1999).

Membrane-associated endothelial NOS is also directly inhibited upon interacting with caveolin-1 (Ju et al., 1997; Feron et al., 1998b; Michel et al., 1997). The N-terminus of caveolin-1 has a cytoplasmic scaffolding domain (a.a. 61-101/82-101) that associates with the caveolin-binding domain (CBD) of eNOS (a.a. 310-570/350-358) at the FSAAPFSGW motif (Garcia-Cardena et al., 1997). This interaction inhibits eNOS

activity, but is reversible by Ca^{++}-calmodulin (Garcia-Cardena et al., 1997; Michel et al., 1997; Ju et al., 1997). The CBD is found between the heme and calmodulin binding domains and is adjacent to a glutamate residue (361) which is necessary for binding L-arginine. It is therefore suspected that caveolin interferes with heme iron reduction (Chen et al., 1997; Frey et al., 1994; Abu-Soud et al., 1994). Although iNOS is a cytosolic protein, studies have found that iNOS, like n- and eNOS, has a caveolin-binding site which, upon association with the caveolin scaffolding domain, leads to inhibition of its activity (Garcia-Cardena et al., 1997). The functional significance of this is not known, although it may simply reflect the conservation of the amino acid sequence between the NOS isoforms.

Acylation sites found within eNOS are important for correct membrane targeting and localization (Sessa et al., 1993). It has previously been found by Liu and Sessa (1994) that eNOS resides primarily on the Golgi (perinuclear region) and in plasma membrane caveolae (on cell edges) of endothelial cells (Liu et al., 1997; Garcia-Cardena et al., 1996). An irreversible, co-translational myristoylation of the amino acid sequence MG^2XXXS (Resh et al., 1994), is unique only to eNOS, and required for membrane localization. Palmitoylation is another form of eNOS acylation required for targeting eNOS to caveolae. This reversible, post-translational modification occurs at cysteine 15 and cysteine 26 and is conditional on the presence of the $(GL)_5$-rich region in between (Liu et al., 1997). Mutation studies have demonstrated that the first 35 amino acids, comprising a region which includes both acylation sites, are necessary for targeting of eNOS to Golgi regions and for basal levels of NO production. This suggests that

palmitoylation is necessary to target eNOS to the trans-Golgi network and then to caveolae in order to produce NO locally in response to hemodynamic forces and activation of cell surface receptors (Garcia-Cardena et al., 1996).

The predominantly membrane-associated eNOS translocates to the cytosol in response to agonists such as bradykinin (BK), cholinergic agents like carbachol that mimic acetylcholine (ACh), and estradiol (Robinson et al., 1995; Feron et al., 1998b; Goetz et al., 1999b). This agonist-induced translocation occurs without the loss of the myristate moiety on eNOS. It was thought that reversible modifications, such as phosphorylation and changes in palmitoylation stimulated by agonists, were the determinants of subcellular eNOS localization. However, it was reported that phosphorylation occurs after eNOS translocation to the cytosol (Michel et al., 1993; Robinson et al., 1995). Translocation of eNOS in response to an agonist is thought to be a mechanism for uncoupling enzyme activation from cell receptor stimulation or to possibly influence the formation and/or release of NO to a further intracellular site (Michel, 1999).

Bradykinin was found to cause depalmitoylation of eNOS (Wedegaertner et al., 1994; Degtyarev et al., 1994), which was then suspected to release eNOS from its inhibitory complex with caveolin and allow translocation. However, it was noted that a rise in Ca^{++} precedes translocation (Goetz et al., 1999b). Interestingly, agonists such as ACh, BK and estradiol cause intracellular Ca^{++} mobilization. Muscarinic (M_2), bradykinin (B_2) and estrogen receptors can be targeted to caveolae upon stimulation by their respective agents to cause elevated Ca^{++} concentration in the cell (Michel et al., 1999; Goetz et al., 1999b;

Isshiki et al., 1999). This eNOS localization to caveolae may improve the efficiency of coupling between eNOS and agonist (Feron et al., 1998a).

2.3.4 Nitric Oxide

As discussed in 2.1.2.4.2, NO is a biologically active free radical gas with a short half-life (3-5 seconds) that can act as a potent vasodilator, inhibit platelet aggregation and adherence (Luscher, 1993), and inhibit leukocyte adhesion (Arnal et al., 1999). It inhibits SMC proliferation (Arnal et al., 1999) and migration possibly by decreasing metalloproteinases (MMP-2 and -9) or by increasing tissue inhibitors of metalloproteinases (TIMPS-2) (Gurjar et al., 1999). NO can also exert cytotoxic effects important in the defense against infection, tumors or various pathogens when iNOS is induced by cytokines such as TNF-α, IL-1β, IL-8, IFN-γ and LPS (Bhagat and Vallance, 1999; Stewart and Marsden, 1995). NO diffuses from endothelial cells to VSMCs or can act within the SMCs directly through activation of soluble guanylyl cyclase (GC) to stimulate increases in the secondary messenger, cGMP (Sanders et al., 2000). This increase in cGMP and associated decrease in intracellular free calcium (Kai et al., 1987; Lincoln and Cornwell, 1993; Twort and Breemen, 1988; Archer et al., 1994; Bolotina et al., 1994) through cGMP-dependent protein kinase and protein phosphorylation, is one mechanism through which NO is thought to exert its effects (Ignarro, 1996), although there is also evidence for cGMP-independent mechanisms. NO inhibits mitogenesis in VSMCs by regulating progression through the cell cycle at G_1 and S-phase via cGMP-dependent and independent mechanisms, respectively (Sarkar et al., 1997a and b). The ability of NO to block mitogenic stimulation of VSMCs, and the potential for

dysregulation of the normal controlling mechanisms may play a part in SMC hyperplasia in conditions such as hypertension and restenosis (Sarkar et al., 1997b).

NO can also decrease the number of Ang II receptors in cultured rat VSMCs in a cGMP-independent manner without changing the affinity for the octapeptide hormone (Cahill et al., 1995). Ang II, a potent vasoconstrictor and mitogenic agent, has also been found to stimulate release of NO and cause endothelium-dependent vasodilation in porcine ECs, mediated partly by the stimulatory effect of its metabolite Ang IV on Ang IV receptors (Hill-Kapturczak et al., 1999).

2.3.5 Dysfunction of NO/NOS in Vascular Disease

With coronary artery disease continuing to be the leading cause of death in North America, targeting factors that lead to or exacerbate these diseases is critical. Agents that regulate NOS could have multiple or pathological effects by altering its ability to produce NO. Elevated NO levels are associated with the rapid drop in blood pressure caused by endotoxin or septic shock (Vane et al., 1990; Broner et al., 1993; Busse et al., 1990), in contrast to the decreased levels of NO which are associated with hypoxia (McQuillan et al., 1994; Liao et al., 1995) and vascular diseases such as atherosclerosis (Hayashi et al., 1992).

SMCs contribute to intimal hyperplasia through migration, proliferation and increased matrix synthesis (Powell et al., 1996; Owens, 1996b), all thought to play a major role in restenosis, post-angioplasty. Expression of MMPs, enzymes that influence

cardiovascular remodeling through their ability to degrade ECM, was found to be induced in human coronary circulation post-angioplasty (Hojo et al., 2002). Endogenous NO inhibits formation of arterial lesions after balloon-angioplasty (McNamara et al. 1993) by preventing SMC migration and proliferation, reducing MMP activity, and preventing adhesion onto cell surfaces. These actions of NO indicate that a defect in the NO/NOS pathway can promote abnormal vascular remodeling and exacerbate pathological vessel wall morphology. NO can balance vasoconstrictors such as norepinephrine and endothelin-1 (Luscher, 1993; Marsden et al., 1991), as well as work synergistically with prostacyclin as an anti-proliferative or anti-thrombic agent. This is of particular importance in endothelial dysfunction or denudation as both states attract platelets and monocytes which release potent proliferative agents, TGF-β1 and PDGF (Luscher, 1993). In addition, an increase in the interaction between ECs, platelets and monocytes can cause endothelial dysfunction. Studies have found that infusing Ang II, an agent thought to contribute to both physiological and pathological effects, causes endothelial dysfunction by increasing NADPH oxidase-mediated vascular superoxide production through eNOS-uncoupling, leading to impaired NO/cGMP signaling (Mollnau et al., 2002). Pueyo and colleagues also found that Ang II stimulated production of peroxynitrite in ECs (Pueyo et al., 1998) and likewise, chronic inhibition of NO synthesis in rats increased vascular oxidative stress via Ang II mediated effects (Kitamoto et al., 2000). It has been suggested that eNOS may represent an important mechanism to up-regulate superoxide dismutase (SOD) expression in VSMCs to prevent superoxide-mediated degradation of NO as it passes from the EC to SMC (Fukai et al., 2000). Therefore, eNOS can exert both a positive and negative effect on the vasculature directly

or by its interaction with a wide variety of factors. Research is ongoing in an effort to examine, elucidate and understand further its role in vascular disease processes.

To review, this body of work examines whether eNOS is expressed in medial VSMCs of porcine LADCAs. In addition, the study will determine if eNOS protein and/or mRNA can be detected in VSMCs from normal and balloon-injured vessels or cultured explants. Also, does Ang II or serum stimulation effect eNOS expression levels? Lastly, is medial eNOS functionally active?

3. MATERIALS AND METHODS

3.1 Materials

Tissue cultures materials were obtained from GibcoBRL (Burlington, ON) (Dulbecco modified Eagle media (DMEM) with 4500 mg/L glucose, liquid antibiotic-antimycotic (AB/AM), penicillin/streptomycin, qualified fetal bovine serum (FBS), 2.5% trypsin, Nunclone plasticware such as 96-well, 90 and 140 mm tissue culture plates), ICN/Flow Technologies (Costa Mesa, CA) (Linbro multi-well culture dishes) and Fisher Scientific (Nepean, ON) (Superfrost glass sides, Costar cell lifter). Chemicals, growth factors and inhibitors/cofactors were purchased from Sigma Chemical Co. (St. Louis, MO) (Angiotensin II, insulin, transferrin, L-ascorbic acid, selenium, tetrahydrobiopterin (BH_4), β-NADPH, L-NAME, protease inhibitors (phenylmethylsulfonylfluoride (PMSF), sodium orthovanadate ($NaVO_3$), sodium fluoride (NaF), aprotinin, leupeptin), dithiothreitol (DTT)), whereas the Ang II receptor antagonists losartan and PD123319 were obtained from DuPont Merck (Rahway, NJ) and Parke-Davis (Ann Arbor, MI) respectively.

Antibodies purchased from Sigma Chemical Co. (St. Louis, MO) were used to detect smooth muscle α-actin and smooth muscle myosin heavy chain (SM1 + SM2 isoforms) (monoclonal), von Willebrand Factor (vWF) (polyclonal), while secondary Cy-2 and Cy-3 conjugated anti-mouse and anti-rabbit antibodies were from Jackson ImmunoResearch Laboratories (BioCan Scientific, Mississauga, ON). Other antibodies used in these studies were: anti-von Willebrand Factor (Boehringer-Mannheim/Roche Diagnostics, Laval, Quebec), anti-smoothelin monoclonal antibody (Chemicon International,

Temecula, CA) and anti-human smooth muscle myosin heavy chain (SM1 + SM2 isoforms) monoclonal antibody (DAKO, Carpinteria, CA). NOS3 (C-20, sc-654) affinity-purified rabbit polyclonal antibody and its blocking peptide were purchased from Santa Cruz (Santa Cruz, CA). Monoclonal anti-caldesmon antibody, purified anti-eNOS monoclonal antibody and anti-rabbit polyclonal antibody were obtained from Calbiochem/Cedarlane Laboratories Ltd (Hornby, ON). Antibodies purchased from Transduction Laboratories/BD PharMingen (Mississauga, ON) were: monoclonal eNOS and affinity-purified polyclonal rabbit antibodies raised against the C-terminus human eNOS immunogen (1030-1209), monoclonal nNOS and iNOS antibodies raised against the C-terminus immunogen (1095-1289) and (961-1144) from human and mouse nNOS and iNOS, respectively. Positive controls (EC, rat pituitary, mouse macrophage lysate) were provided with Transduction/Pharmingen antibodies. Dilutions used for primary antibodies are listed in Table 1.

Other purchases included bovine serum albumin (fraction V) and Triton X-100 (Boehringer-Mannheim/Roche Diagnostics, Laval, Quebec), Tween-20 (BioRad, Richmond, CA), Streck fixative (Streck Labs Inc., Omaha, NE), paraformaldehyde (TAAB Laboratories Equipment Limited, Aldermaston, England) and bisbenzimide H 33342 fluorochrome trichloride /Hoescht 33342 nuclear stain (Calbiochem/Cedarlane Laboratories Ltd, Hornby, ON). Additional reagents used were Crystal mount anti-fade mounting media (Biomedia Corp., Foster City, CA), tissue-tek O.C.T. (optimal cutting temperature) embedding compound (Sakura Finetek USA Inc., Torrance, CA). Immobilon-P polyvinylidene difluoride (PVDF) membranes were obtained from

Millipore; the ECL chemiluminescent detection system and VISTRA Green DNA stain were purchased from Amersham Pharmacia Biotech (Oakville, ON) and ethidium bromide was from Sigma Chemical Co. (St. Louis, MO).

LADCAs were rapidly dissected from porcine hearts obtained from the local abattoir. The coronary/balloon angioplasty catheters of various sizes used in these studies were obtained from Boston Scientific/SciMed (Maple Grove, MN) or Cook Inc. (Stouffville, ON). TRIzol, used for extracting total RNA, was from GibcoBRL (Burlington, ON), RT-PCR kits (Promega) were distributed by Fisher Scientific (Nepean, ON), NOS Assay Kits (catalogue no. 781001) were from Cayman Chemical (Ann Arbor, MI) and (catalogue no. 482702) Calbiochem-Novabiochem Corp. (San Diego, CA) and the TOPO cloning kit with pCR®2.1-TOPO® vector (plasmid) was from Invitrogen (Burlington, ON). [^{3}H]-arginine monohydrochloride was purchased from Amersham Pharmacia Biotech UK Ltd. (Oakville, ON), while Aquasol, Cytoscint and EcoLume were from DuPont-NEN/Mandel-NEN (Guelph, ON) and ICN/Flow Technologies (Coast Mesa, CA), respectively.

3.2 Methods

3.2.1 Experimental Systems:

3.2.1.1 Porcine Vascular Smooth Muscle Cell Cultures

Primary cultures of vascular smooth muscle cells (VSMCs) were generated from free-floating explants of the left anterior descending coronary artery (LADCA) according to Saward and Zahradka (1997a). Hearts were obtained from juvenile pigs 6-12 months of age and transported from the local abattoir on ice. The LADCAs were carefully dissected out with fine forceps and scissors, cut into 2-5 mm segments and placed into culture with high glucose Dulbecco's modified Eagle medium (DMEM) containing 20% fetal bovine serum (FBS), 50 μg/ml streptomycin, 50 μg/ml penicillin and either 1× or 10× antibiotic-antimycotic (AB/AM) (10,000 units/ml penicillin G sodium, 10,000 μg/ml streptomycin sulfate, and 25 μg/ml amphotericin B as Fungizone® in 0.85% saline). The VSMCs begin to migrate from the explants approximately two weeks after being placed into culture. The second wave of cells from the explanted tissue was released from the dish by trypsinization (0.2% trypsin) after reaching approximately 75% confluence, and the population expanded over 2 passages. Cells were subsequently seeded onto experimental plates with media being changed every 2-3 days (see Table 2 for the density of cells plated). Once the cells reached 75% confluence (within 1-2 days), the growth medium was removed, the cells were rinsed twice with either PBS (0.9% NaCl, 0.1 M $NaPO_4$ pH 7.1) or serum-free DMEM, and differentiation media (DMEM supplemented with 11 μg/ml pyruvate, 5 μg/ml transferrin, 10^{-9} M selenium, 2×10^{-4} M ascorbate and 10^{-8} M insulin) was added. The cells were maintained under these conditions for 5-7 days.

3.2.1.2 Organ culture

Size-matched pig hearts weighing approximately 440 grams were obtained from the local abattoir and transferred on ice to the laboratory within 30 minutes. The proximal and distal ends of the LADCA were cut to permit gentle flushing with PBS containing 10× AB/AM. The superficial, anterior aspect vessel was then exposed by dissection. A balloon-angioplasty catheter (3.5-4.0 mm diameter, 20-30 mm length) was inserted into the artery to a point immediately distal to the first major diagonal branch off of the LADCA. The balloon was inflated to 6 atmospheres for 1 minute. The vessel was then flushed with PBS and approximately 2 cm of the vessel dissected completely out. Control vessels were treated similarly excluding the balloon angioplasty. The vessels were then cut into equal 5-mm segments or left intact and either placed randomly into various wells labeled injured or non-injured, or placed into wells labeled according to each individual pig heart. These dishes were incubated at 37°C in 5% CO_2 in 1 ml DMEM with 20% FBS, 50 μg/ml streptomycin, 50 μg/ml penicillin and 10× AB/AM. Media and treatments were replaced every 2-3 days (Wilson et al., 1999). The vessel segments were maintained in culture for one to five days. Once the appropriate number of days had passed, the vessels were placed into O.C.T. compound without prior fixation, frozen in a dry ice-ethanol mixture (bottom first, followed by the rest of the block) and stored at -80°C until ready for cryosectioning (Del Rizzo et al., 2001).

3.2.2 Medial (VSMC) Preparations from Porcine Coronary Arteries

Medial preparation involved the careful excision of porcine coronary arteries followed by the precise removal of the medial layer from this vessel. A vessel may be injured by

inflation of a balloon-catheter prior to its excision from the heart or isolated without injury. The LADCA were isolated according to the procedure described previously (3.2.1.2) and placed into a conical tube containing ice-cold PBS and 10× AB/AM. Fresh drapes were laid down over a clean surface e.g. bench top or surgical table. Labeled sterile 1.5 ml microfuge tubes were placed on ice and filled with 1.0 ml PBS with 10× AB/AM. A folded sheet of aluminum foil was placed on an ice-pack or bed of ice. The intact vessel was removed from the conical tube or from culture with clean forceps and placed on top of the foil. Without stretching the vessel, it was placed flat and cut length-wise so that the endothelial layer faced upward. With a gentle sweeping motion, the endothelium was lightly scraped off with a sterile scalpel blade while being careful to include the grooves along the vessel (side-branches). The vessel was then gently rinsed numerous times with ice-cold PBS containing 10× AB/AM. With a fresh blade and while holding one end of the vessel with forceps, a thin length-wise slit was made down the vessel after making two thin slits across the top and bottom of the vessel, 2 mm from the ends. While holding the vessel with one pair of forceps, the medial layer was peeled away with the other pair of forceps. The smooth muscle was removed in neat strips and without much resistance. Any tough fibrous material was considered adventitia and therefore discarded. The peeled strips were immediately placed into microfuge tubes containing ice-cold solution. Samples from the same treatment conditions were pooled in the same microfuge tube. The tubes were centrifuged cold (4°C) at 10,000-12,000 rpm (Microfuge® 18 centrifuge, Beckman) for 5 minutes. The PBS was removed, the tissue immediately frozen in liquid nitrogen and stored at -80°C until ready to use.

3.2.3 Histology

3.2.3.1 Cryosectioning

Blocks of the vessel sections frozen in OCT (taken directly from the -80°C freezer) were mounted on chucks and cut with a cryostat (Kryostat 1720 Digital, Leitz). The first 1.0-1.5 mm of the cut surface was removed. Transverse 7.0 μm sections were placed onto Superfrost slides and stored at -80°C until ready for use (Wilson et al., 1999).

3.2.3.2 Cell/Tissue Fixation

Paraformaldehyde was prepared in advance: Four ml of 9% NaCl, 16.0 ml ddH_2O and 70 μl 1N NaOH were heated to 55°C in a microwave, then 1.6 g paraformaldehyde was added with stirring until dissolved. Finally, 8.0 ml 0.5 M $NaPO_4$ pH 7.4 and 12 ml reverse osmosis purified water (RO H_2O) were added to the final mixture, after which the solution was filtered through a 0.22 μm cellulose acetate membrane (Corning Costar, N.Y.). The paraformaldehyde solution was stored at 4°C and used within 24 hours. Slides were removed from culture and washed 3× with PBS, whereas immunohistochemistry slides were removed from -80°C and warmed to room temperature prior to fixation. The slides were then placed into coplin jars containing cold 4% paraformaldehyde for 10 minutes. Slides were rinsed 3 × 5 minutes with PBS (0.9% NaCl, 0.1 M $NaPO_4$ pH 7.1), permeabilized with cold 0.1% Triton X-100 in PBS for 10 minutes then rinsed for 2-3 × 5 minutes with PBS (Saward and Zahradka, 1997b).

3.2.3.3 Immunofluorescence Microscopy

Slides placed into a humid chamber were blocked in 3% BSA in 1× TBS-T (1 M Tris-HCl pH 7.4, 5 M NaCl, Tween-20®, RO H_20) for 60 minutes at room temperature after which the blocking buffer was gently removed by aspiration. The sections were then incubated with various primary antibodies (see Table 1) for 60 minutes at room temperature after dilution in 3% BSA in 1× TBS-T. Slides were washed thoroughly with PBS/TBS/TBS-T and the primary antibody was visualized after incubation with Cy3-/Cy2-coupled secondary antibody (diluted 1:400 in 1% BSA in 1× TBS-T) for 60 minutes at room temperature in a light-proof box. After a thorough washing, the nuclear stain, Hoescht 33342 (0.5 mg/ml in water, diluted 1:1000 in TBS/TBS-T), was applied for 5 minutes followed by extensive washing with TBS/TBS-T. The backs of the slides were dried and excess fluid was removed with blotting paper before the addition of 1-2 drops of Crystal Mount mounting media (Saward and Zahradka, 1997b).

3.2.3.4 Photography

Images from an Olympus BH-2 RFCA epifluorescence microscope were digitally captured with a DAGE-MTI CCD camera using FlashPoint FPG 3.10 with integrative software (Integral Technologies, Inc., 1997) at a magnification of either 100× or 200×, or photographed with a 35-mm camera using Fuji Provia 400 slide film. Exposures were set manually to allow equal exposure for all photographs taken for each experiment, allowing for gross comparison of immunofluorescence between slides within the same experiment.

3.2.4 Protein Analysis

3.2.4.1 BCA Protein Assay

The BCA protein assay employed a protocol designed for 96-well tissue culture plates. Cell lysates (10 µl, with or without prior dilution) were added to the wells in triplicate alongside an appropriate blank and BSA protein standards (ranging from 0.2-1.0 mg/ml). BCA reagents A and B (Pierce, Rockford, IL) were mixed together (50:1) and 200 µl was added to each well. The plate was incubated at 37°C for 30 minutes, allowed to cool for approximately 5 minutes, and color development was quantified at 550 nm with a Molecular Devices ThermoMAX plate reader. Refer to Pierce BCA Protein Assay Reagent™ instructions for further details.

3.2.4.2 Cell Lysate Preparation

For activity assays, cells plated in 6-well dishes were placed immediately on ice and rinsed twice with PBS containing 1 mM Na_3VO_4, 1 mM NaF and 1 mM PMSF. The cells were lysed by addition of RIPA buffer (50 mM Tris-HCl pH 7.5-8.0, 1% NP-40, 0.25% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA) containing freshly added PMSF (1 mM), aprotinin, leupeptin, pepstatin A (each at 1-2 µg/ml), 1 mM Na_3VO_4 and 1 mM NaF. The protease inhibitors were replaced with a mammalian protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO) for certain experiments. After a 5 minute incubation, each well was scraped and the lysates transferred to microfuge tubes and centrifuged at 12,000×g for 10 minutes at 4°C. The supernatant of each lysate sample was assayed to determine the protein concentration.

Cell lysates for use in Western blotting were prepared with 200 µl 2× SDS-sample buffer (0.125 M Tris-HCl pH 6.8, 2% SDS, 20% glycerol) per well in lieu of RIPA buffer. However, when using sample buffer (SB) to prepare cell lysates, it was necessary to sonicate each of the samples after transfer to microfuge tubes, prior to centrifugation.

3.2.4.3 Immunoblotting (Western blotting)

Cell lysates were mixed with 2× SB (containing bromophenol blue and β-mercaptoethanol) and heated for 5 minutes at 90°C prior to loading onto a 6.0% or 7.5% polyacrylamide gel. Following electrophoresis at a current of 20 mA/gel for 60 minutes, proteins were transferred onto PVDF membrane at 100V (0.5A) over 60 minutes in Tris-glycine (10-15% methanol, 25 mM Tris, 130 mM glycine) transfer buffer. Membranes were blocked in 3% BSA in 1× TBS-T for 60 minutes at room temperature then incubated with primary antibody diluted in (3% BSA in 1× TBS-T) for 60 minutes at room temperature (see Table 1). Membranes were washed 6 × 5 minutes with TBS-T and incubated with an HRP-conjugated secondary antibody (diluted 1:5000-10,000 in 1% BSA in 1× TBS-T) for 60 minutes at room temperature. After washing 6 × 5 minutes with TBS-T, the membrane was incubated in an ECL chemiluminescent reagent (Amersham Pharmacia Biotech, UK) for 5 minutes and subsequently exposed to film (Kodak Scientific Imaging film (X-Omat Blue XB-1), Rochester, N.Y.) (Yau et al., 1999). The density of the bands was quantified using BioRad Model-670 Imaging Densitometer and Molecular Analyst computer software.

3.2.5 Nucleic Acid Manipulation

3.2.5.1 Primer Preparation

Primers were prepared based on the relevant amino acid sequences of proteins from different species including porcine. Sequences downloaded from GenBank (http:// www.ncbi.nlm.nih.gov/) were used in conjunction with the new version of Primer 3 (http:// www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and BLAST (http:// www.ncbi.nlm.nih.gov/BLAST) to design oligodeoxynucleotide (ODN) primers for RT-PCR. Primers were synthesized with an Oligo1000 DNA Synthesizer (Beckman Instruments Inc., Mississauga, Ontario) onto G, T, A and benzoyl-C 30 nM columns (Beckman Instruments Inc., Mississauga, Ontario) according to the manufacturer's instructions. The oligodeoxynucleotides were released with concentrated ammonium hydroxide (1.5 hours at room temperature), deprotected by incubating for 15 hours at 55-60°C and dried under vacuum. The primers were reconstituted in RO H_2O and the absorbance read at 260 nm to determine the amount of oligonucleotide synthesized (1 O.D. unit = 33 μg/ml). Primer sequences (e.g. SM myosin heavy chain, nNOS, iNOS, vWF) are listed in Table 3. However, utilizing only Primer 3 and BLAST programs to design primers (as described above) was insufficient to produce specific porcine eNOS primers. Therefore, endothelial NOS primers were developed by first manually aligning all of the eNOS amino acid sequences from *Bos taurus, Sus scrofa, Canis familiaris, Cavia porcellus, (Rattus norvegicus, Ovis aries, Xenopus)* and *Homo sapien* to identify areas of conservation. The amino acid sequences for human nNOS, iNOS, eNOS and *Sus scrofa* eNOS were then aligned to delineate specific areas unique to eNOS after which selected regions were subjected to Primer 3 and BLAST programs to select the

primers. The primers were then synthesized by GibcoBRL Custom Primers. The rat GAPDH primers were based on the sequence by Wong et al. (1994).

3.2.5.2 RNA Preparation

Total RNA was extracted from cells on 90-mm or 150-mm dishes with TRIzol reagent. The amount of TRIzol reagent used is based on the area of the culture dish, not on the cell number, according to the manufacturer's instructions (Life Technologies, GibcoBRL, Burlington, ON). Cells were rinsed 1-2× with PBS and 1.0 ml TRIzol per 10 cm^2 was added for 5 minutes. Total RNA was also extracted from fresh and frozen (-80°C) medial SMC preparations that was frozen in liquid nitrogen then crushed into powder with a mortar and pestle. TRIzol was added to the powder and the mixture then homogenized (50-100 mg tissue per 1.0 ml TRIzol) three times on ice for 10-15 seconds, using a power homogenizer (Pro 200 homogenizer). The sample was then centrifuged at 12,000×g for 10 minutes at 4°C to remove insoluble material from the homogenate and the supernatant containing RNA was transferred to fresh microfuge tubes (1.0 ml TRIzol-extract per tube). Chloroform (200 µl) was added, the tubes inverted gently to mix and the samples centrifuged at <12,000×g for 15 minutes at 4°C. The clear (upper) aqueous phase was transferred to a fresh tube and mixed with 500 µl (-20°C) isopropanol for 10 minutes at room temperature. Precipitated RNA (white pellet) was collected by centrifugation at 12,000×g for 10 minutes at 4°C, washed with 1 ml (-20°C) 75% ethanol and re-centrifuged at 7500×g for 5 minutes at 4°C. The RNA was air-dried by inverting the tubes for 30 minutes, then reconstituted in 30-50 µl DEPC-treated water. Using a UV/Visible spectrometer (UNICAM 8625, UNICAM Analytical Inc., Toronto, ON), the

absorbance at 260, 280 and 240 nm was used to determine the quantity and purity of the extracted RNA. Empirically, an absorbance (optical density) of 1.0 at a wavelength of 260 nm corresponds to 40 μg/ml of RNA. This information was used to calculate the concentration for each RNA sample. To visualize the RNA (18S and 28S bands) and detect contamination or degradation, equal concentrations (0.5 or 1.0 μg total RNA) for each sample were run on 1.0 % TAE gels, prepared with 1× TAE running buffer (50×: 242 g Tris, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA pH 8.0, brought up to 1 L with DEPC-treated water).

3.2.5.3 RT-PCR

Prior to RT-PCR, contaminating DNA in the RNA samples was removed by incubating 1 μg RNA (extracted from medial tissue) with DNase I for 15 minutes at room temperature. The DNase was inactivated by addition of 1 μl 25 mM EDTA followed by heating for 10 minutes at 65°C. Reverse transcription (RT) of the RNA was conducted according to the instructions provided with the Access RT-PCR System (Promega) as previously described (Del Rizzo et al., 2000). A master mix was made containing 1 μl AMV reverse transcriptase (5U) in 10 μl AMV/*Tfl* 5× reaction buffer (50% glycerol, 20 mM Tris-HCl pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% NP-40, 0.5% Tween-20) with 1 μl dNTP mixture (10 mM each dNTP), 50 pmol of both upstream and downstream primers and 1 μl *Tfl* DNA polymerase (5U). The DNA-free RNA samples (11 μg) were added to the master mix plus DEPC-treated water to a final volume of 50 μl. First strand synthesis (RT) was carried out by incubating for 45 minutes at 48°C, followed by 2 minutes at 94°C to inactivate the reverse transcriptase and allow for RNA/cDNA and

primer denaturation. Second strand synthesis and PCR amplification were carried out over 30 cycles with a 30 second denaturation step at 94°C, a 1 minute annealing step at 62°C and a 2 minute extension step at 68°C. In the final cycle, the extension step was held at 68°C for 7 minutes. Control reactions (no RNA, EC RNA from LADCA, plus primers and RNA provided by Promega) were used with each PCR reaction. The PCR products were analyzed by electrophoresis in 1.7% agarose gel with TBE (5× TBE: 0.045 M Tris-borate, 0.001 M EDTA pH 7.5-7.8) buffer. The bands were visualized with ethidium bromide or VISTRA green nucleic acid stain. Photographs were taken using a Polaroid DS34 Direct Screen Instant Camera fitted with an EP-H6 0.8× hood and an orange or green filter employing Polaroid 667 black and white instant film. Band intensity was quantified by using a BioRad Model-670 Imaging Densitomer and Molecular Analyst computer software (for films) or Amersham Pharmacia Storm Scanner using ImageQuant software (for agarose gels). To establish primer specificity, bands amplified in the presence of the eNOS primers were cloned (using chemically competent *E. coli)* into pCR®2.1-TOPO® vector plasmid (Invitrogen, Burlington, ON) and sequenced by Cortec DNA Service Laboratories, Inc. (Queens University).

3.2.6 Activity Assays

The NOS assay kit (Cayman Chemical, Ann Arbor, MI) is based on the conversion of L-[2,3,4-^{3}H] arginine to neutral L-citrulline by a cell extract, and a protocol was provided by the manufacturer. Quiescent SMCs were stimulated for 2.5 minutes with 10^{-5} M bradykinin. The cells were then rinsed twice with cold PBS containing 1 mM EDTA, collected and snap-frozen in liquid nitrogen and stored at -86°C. Later, proteins were

extracted from cells resuspended in 100 µl homogenization buffer (10×: 250 mM Tris-HCl pH 7.4, 10 mM EDTA, 10 mM ethyleneglycol-bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA)) after brief sonication and centrifugation at full speed (13,500 rpm, Microfuge®18 Centrifuge, Beckman) for 5 minutes. Similarly, tissue extracts were prepared by disrupting the tissue in homogenization buffer and collecting the supernatant (soluble) or pellet (particulate) fractions by centrifugation at full speed (13,500 rpm, Microfuge ® 18 Centrifuge, Beckman) for 5 minutes. The protein concentration of each sample was adjusted with homogenization buffer to 5-10 µg/ml. A master mix of the reagents, 2× reaction buffer (50 mM Tris-HCl pH 7.4, 6 µM tetrahydrobiopterin (BH_4), 2 µM flavin adenine dinucleotide and 2 µM flavin adenine mononucleotide), 10 mM freshly prepared β-NADPH, 6 mM $CaCl_2$, 1 µCi/µl [^{3}H]-arginine and 1 µM calmodulin were prepared and 45 µl was added to the cell samples. The positive control (rat brain extract) and the NOS inhibitor, L-NAME, were provided with the kit. The samples were incubated at room temperature or in a 37°C waterbath for 60 minutes and the reaction was stopped with 400 µl stop buffer. The samples were resuspended in 100 µl equilibrated ion-exchange resin, mixed and transferred to spin cups. The samples were centrifuged at full speed for 30 seconds, the flow-through collected and an aliquot (50 µl) was placed into scintillation vials with 20 ml Aquasol or EcoLume scintillation fluid. An additional 400 µl elution buffer was added to each spin cup after the original flow-through was collected, and the cups were centrifuged a second time for 30 seconds. A 50 µl aliquot was treated like the first flow through. The radioactivity was quantified with a scintillation counter (Beckman Instruments Inc., Mississauga, Ontario). The ratio of unreacted L-arginine (first flow-through) versus converted L-arginine (L-citrulline, the

second eluted flow-through) was indicative of activity, and can be determined by the formula:

(First flow-through / Eluted flow-through) × 100

3.2.7 Data measurement and statistical analysis

The GraphPad Prism analysis program (GraphPad Software, Inc. (1999)) was used to perform a parametric one- and two-way analysis of variance (ANOVA) on Western blot data obtained by densitometry. The Kruskal-Wallis test, a non-parametric test which unlike the parametric test does not assume a Gaussian distribution of data, was also performed, however, no post-test (e.g. with Mann-Whitney test, a non-parametric equivalent of the t-test) was performed (refer to pages 110-112 in the discussion). Dunnett's Multiple Comparison test was used as the post-test to compare one control group to all other groups if significance ($p<0.05$) was detected with ANOVA. A confidence interval for differences between all pairs of means was set at 95% ($p<0.05$).

Table 1 – Dilution of primary antibodies used for immunostaining

Antibody	Source	Dilution	
		(IF)	(WB)
eNOS (m)	TL	1:250	1:2500
ECNOS (p)	TL	1:100	1:1000
nNOS (m)	TL	1:250	1:2500
iNOS (m)	TL	1:1000	1:10000
anti-eNOS (m)	Calbiochem	1:50	1:500
anti-iNOS (p)	Calbiochem	1:500	
caldesmon (m)	Calbiochem	1:100	
NOS III (p)	SC	1:500	1.5:1000
smoothelin (m)	Chemicon	1:100	1:5000
SM myosin heavy chains (m)	DAKO	1:100	1:1000
SM myosin heavy chains (m)	Sigma	1:2500	
SM actin (m)	Sigma	1:1000	1:5000
von Willebrand factor (p)	Sigma	1:25	1:500
von Willebrand factor (m)	Roche	1:10	1:100

Key
TL - Transduction Laboratories
SC - Santa Cruz
IF - Immunofluorescence
WB - Western blotting
m - monoclonal
p - polyclonal

Table 2 - VSMCs plated at the following densities

Dish diameter	Cells/dish
140 mm plates/slides	10-15 x 10^5 cells/plate
90 mm plates	5.0-10 x 10^5 cells/plate
6-well plates	1.0-2.0 x 10^5 cells/well
12-well plates	0.5-1.0 x 10^5 cells/well
24-well plates	0.25-0.5 x 10^5 cells/well

Table 3 - Primers used for RT-PCR amplification

Smooth muscle myosin heavy chain (accession number AF020091) with an expected product size of 303 bp

Upstream - 5' CCG ACT CGA AGA AGA AGC TG 3'
Downstream - 5' CAG CTC TGT CCC TCT CAT CC 3'

eNOS/NOS III (accession numbers M95296 (human), U59924 (porcine)) with an expected product size of 364 bp

Upstream - 5' CCT GCA CTA TGG AGT CTG CTC 3'
Downstream - 5' GAT GTC CTG CAC GTA GGT CTT AG 3'

nNOS/NOS I (accession number U31466) with an expected product size of 265 bp

Upstream - 5' CAT GGA GGA TCA CAT GTT CG 3'
Downstream - 5' GGC AAT GCC TCT GAG TAC CT 3'

iNOS/NOS II (accession number U31511) with an expected product size of 221 bp

Upstream - 5' GTG GAA GCG GTA ACA AAG GA 3'
Downstream - 5' TTG CCA TTG TTG GTG GAG TA 3'

von Willebrand Factor/vWF (accession number AF051036) with an expected product size of 289 bp

Upstream - 5' TTC TTG ACC TGG TCT TCC TG 3'
Downstream - 5' CTG CCG AAG ATT TGG AAG AG 3'

rat GAPDH (reference: Wong et al., 1994) with an expected product size of 306 bp.

Upstream - 5' CGC TGT GAA CGG ATT TGG CCG TAT 3'
Downstream - 5' AGC CTT CTC CAT GGT GGT GAA GAC 3'

4. RESULTS

4.1 eNOS Expression in Porcine LADCAs Post-Angioplasty

eNOS has been detected in cell types other than ECs including human and rabbit gastrointestinal SMCs (Sase and Michel, 1995; Teng et al., 1998). The possibility that eNOS was present in VSMCs was explored using an isolated organ culture model, according to the procedure of Wilson et al. (1999). Left anterior descending coronary arteries (LADCAs) were either subjected to balloon-angioplasty or not, prior to excision (see Materials and Methods 3.2.1.2) and examined after being placed into organ culture for 24 to 96 hours. By using the same exposure times to examine each slide within the same experiment, differences in eNOS staining with a monoclonal anti-eNOS antibody (Calbiochem) were visible between the 48 hour non-injured and the balloon-injured coronary artery sections (Figure 2). Non-injured vessels displayed high level eNOS staining within the endothelial monolayer (panel A). In contrast, eNOS staining of the denuded balloon-injured vessels was visible within the medial VSMC layer (panel C). The low-level, non-uniform pattern of staining was not an artifact of the culture system, as eNOS was limited to the endothelium of non-injured vessels at both 0 and 48 hours of culture (Figure 3, panels A and E).

4.1.1 Altered eNOS Expression in Sections Over Time

Immunohistochemical staining (IH) showed that eNOS expression in the tunica media increased over time, post-angioplasty (Figure 4). Following placement of porcine LADCA into culture, non-uniform eNOS expression within the medial layer gradually increased. This is evident at the 48 hour time point (panel C) where there was no medial

Figure 2: Immunohistochemical Staining of eNOS in Porcine LADCAs

Porcine coronary artery sections placed on positively-charged Superfrost slides were fixed with Streck fixative and stained with anti-mouse eNOS antibody (Calbiochem) (A,C) at a dilution of 1:50 as described in Materials and Methods. The sections were also counterstained with Hoescht 33342 (B,D) to visualize the nuclei. Micrographs of non-injured (A, B) and balloon-injured (C, D) artery segments are shown after 48 hours in culture. The location of the endothelial (E) and medial (M) layers are indicated. Magnification: 250×

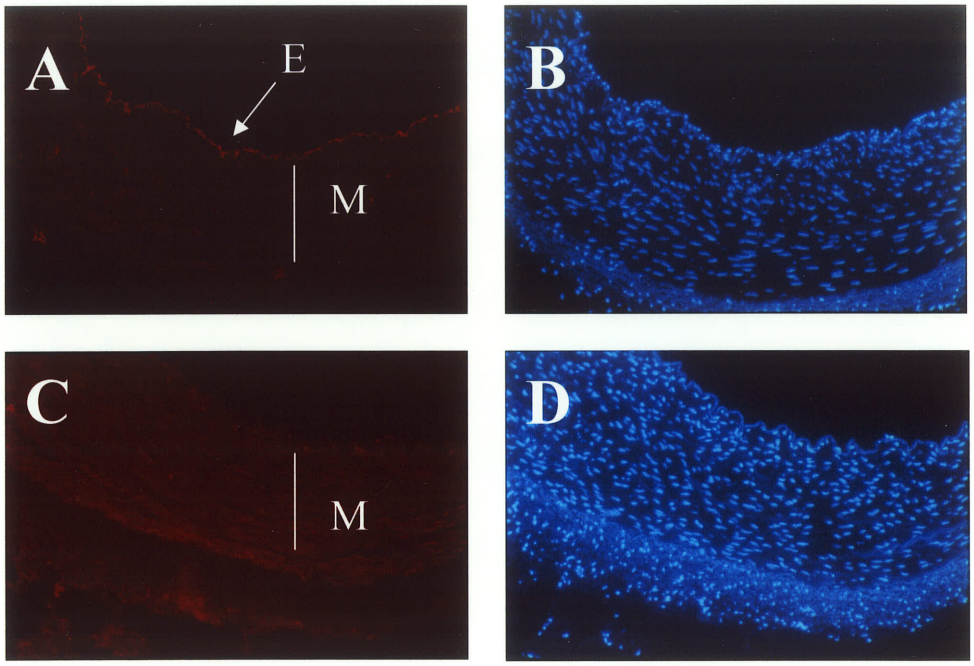

A
E
M
B
C
M
D

Figure 3: Comparison of eNOS Localization in Balloon-injured versus Non-injured LADCAs

Non-injured (A,B,E,F) and balloon-injured (C,D,G,H) LADCAs were sectioned immediately (A-D) and after 48 hours of organ culture (E-H) onto positively-charged Superfrost slides. Slides were fixed with Streck, stained with both NOS III (Santa Cruz) antibody (A,C,E,G) at a dilution of 1:500 and Hoescht 33342 (B,D,F,H). Magnification: 250×

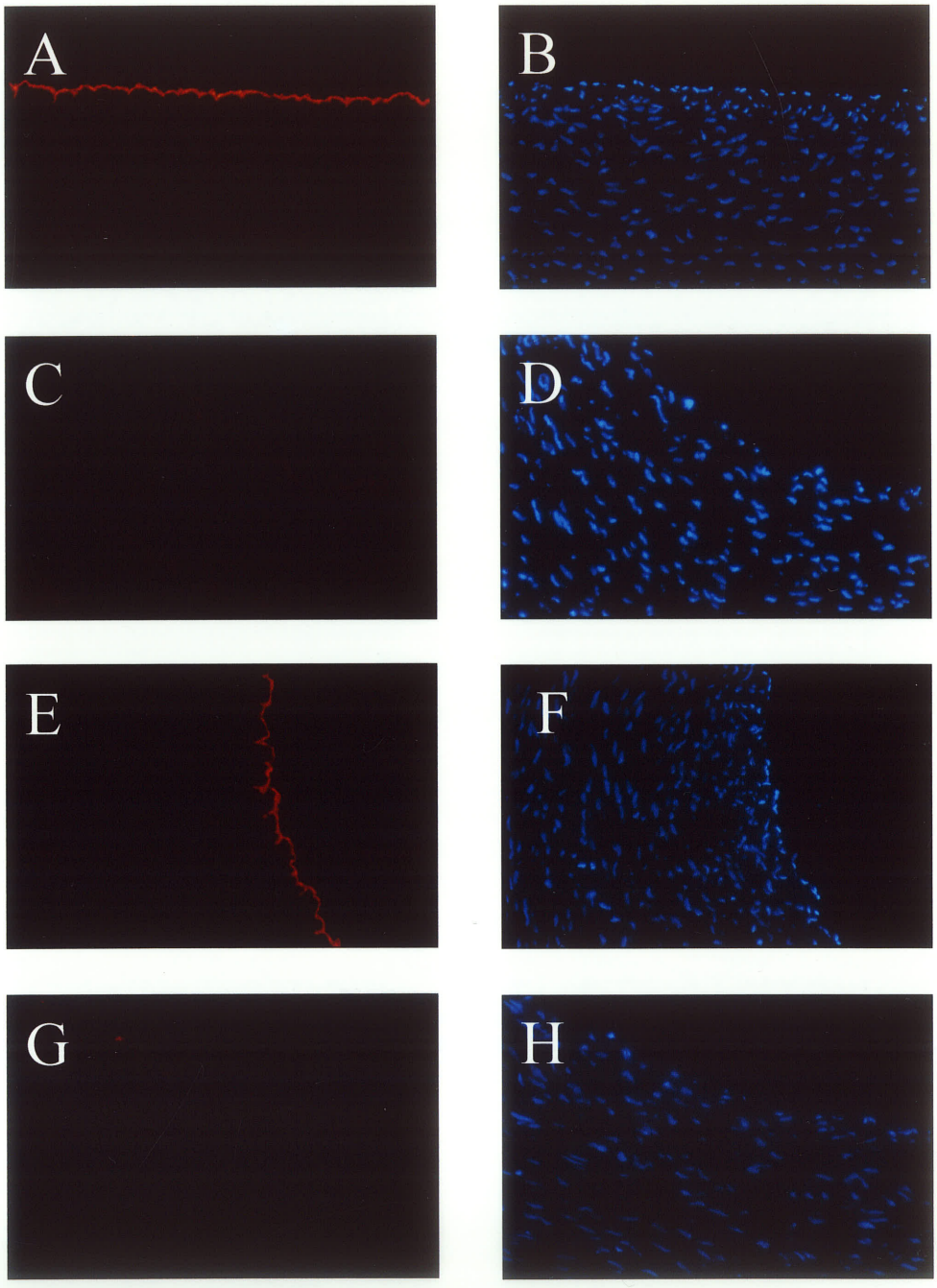
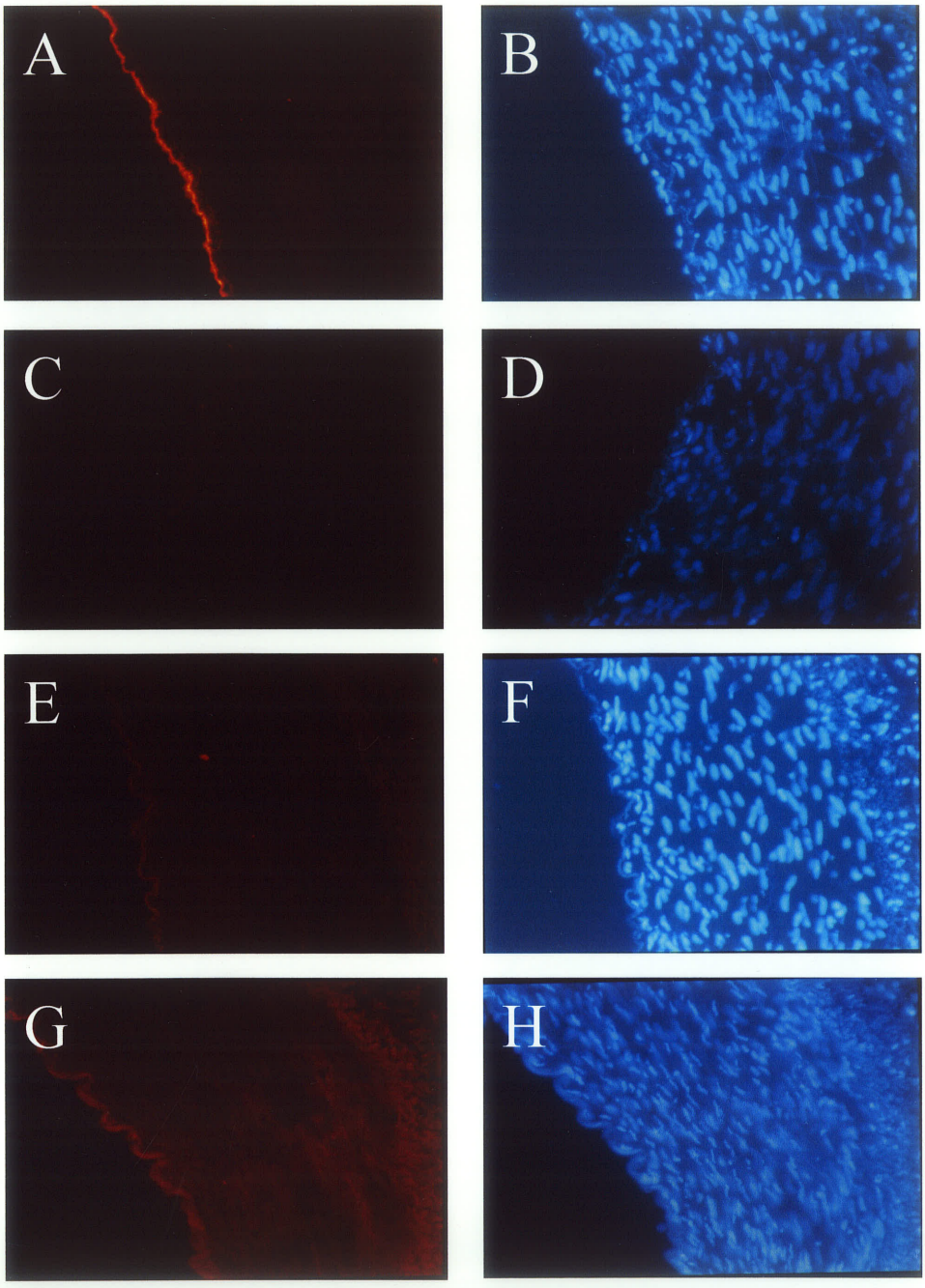

A
B
C
D
E
F
G
H

Figure 4: Altered eNOS Staining Over Time in LADCAs Post-Angioplasty

Porcine LADCA were removed from culture at various time points and stained with polyclonal NOS III (Santa Cruz) antibody (A,C,E,G) at a dilution of 1:500 and Hoescht 33342 (B,D,F,H). The micrographs show: 0 hr non-injured (A,B), 48 hr (C,D), 53 hr (E,F) and 72 hr (G,H) balloon-injured vessels. Similar results were obtained in two independent experiments using this antibody. Magnification: 250×


A
B
C
D
E
F
G
H

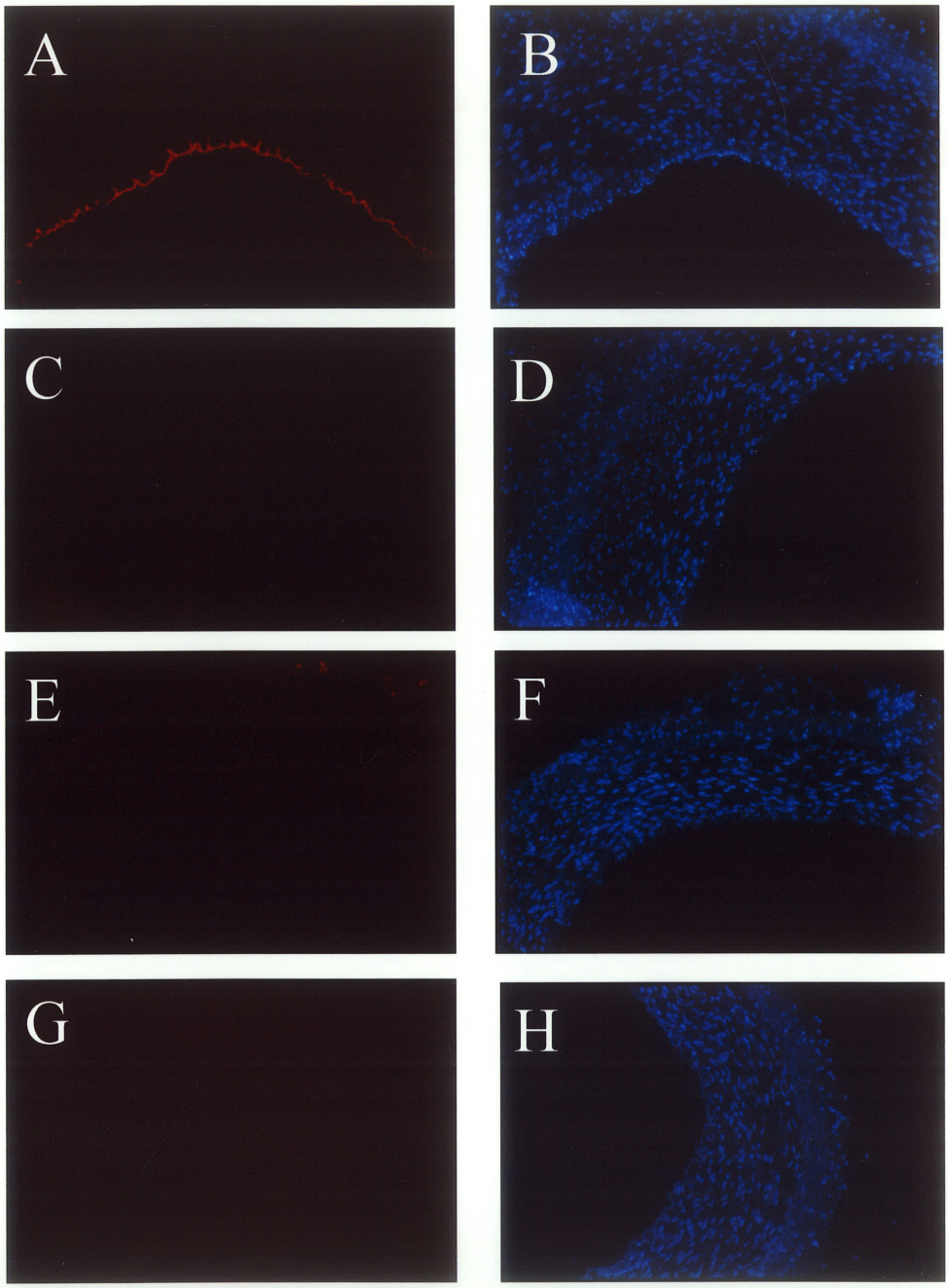
eNOS staining with the polyclonal anti-NOS III antibody (Santa Cruz (SC)), however, eNOS was detected in various coronary artery segments at 53 hours (panel E) and 72 hours (panel G) post-injury. Control sections displayed typical eNOS expression within the ECs (panel A).

4.1.2 Variability of eNOS Staining

Although the data shown in Figures 2-4 were reproducible, there were times when eNOS staining could not be detected within the tunica media (Figure 5). This variability was observed when sections from different porcine samples were stained with the same antibody (within the same or different experiments), as well as when antibodies from different sources (polyclonal anti-NOS III (SC), polyclonal anti-ECNOS (Transduction Laboratories (TL)), monoclonal anti-eNOS (Calbiochem)) were employed. The SC antibody was consistently unable to detect eNOS expression in the medial layer at 48 hours (Figures 3G, 4C and 5E) but able to detect eNOS at 53 and 72 hours (Figure 4E, G) post-injury. This Santa Cruz antibody was a polyclonal affinity-purified antibody which was tested with ECs as a positive control. Although the same antibody was used, eNOS expression in the media was not always detected at the later time points (Figure 5G).

The Calbiochem antibody detected the protein within the medial layer at 48 hours (Figure 2C), however, there appeared to be individual variability between different porcine LADCAs. Thus, it could be stated that eNOS expression was present within medial sections, however, the time point at which eNOS was detected was not consistent. Due to this variability, Western blot analysis was used to confirm the immunohistochemistry

Figure 5: eNOS Expression Post-Angioplasty
Balloon-injured porcine LADCA were stained with NOS III (Santa Cruz) antibody (A,C,E,G) at 1:500 dilution and Hoescht 33342 (B,D,F,H). The micrographs show: 0 hr non-injured (A,B), 24 hr (C,D), 48 hr (E,F) and 72 hr (G,H) balloon-injured vessels. Magnification: 125×


A
B
C
D
E
F
G
H

data showing expression of eNOS within the medial VSMC layer.

4.2 eNOS Present in PCA Medial Preparations

Medial preparations from denuded porcine LADCAs were obtained over 24 hour intervals, extending from 0 to 96 hours after balloon-injury, and cell lysates were prepared from pooled samples. Western blot analysis using polyclonal anti-NOS III antibody (SC) detected a 140 kDa protein in medial VSMCs samples (Figure 6). The highest expression was observed in samples prepared from uncultured vessels immediately post-injury (panel A, lane 1). After being placed into culture, the samples (lanes 2-5) showed a decrease in eNOS levels within 24 hours to a level that remained low for all later time points. These data confirm the presence of eNOS in the medial layer. Repeated experiments showed similar results, although the differences between the 0 hour (non-cultured) and cultured samples were not always as great (panel B).

Detection of eNOS in medial preparations by Western blotting was reproducible with the polyclonal SC antibody. Neither iNOS nor nNOS were detected in medial samples with monoclonal anti-iNOS/nNOS antibodies (TL) (data not shown). As with immunohistochemistry, the ability to detect eNOS was dependent upon the antibody used (Figure 7). Medial eNOS was detected with the polyclonal anti-NOS III antibody from SC. In contrast, the monoclonal anti-eNOS antibody from TL failed to detect medial eNOS under the same conditions, although an eNOS band could be seen on a longer exposure (Figure 8). The TL antibody required an exposure time of 10 minutes

Figure 6: Western Blot Analysis of eNOS Expression in Medial Preparations

Medial extracts were prepared from balloon-injured coronary arteries placed into culture for varying time periods. The relative amount of eNOS (140 kDa band) in the extracts was assessed by Western blot analysis. The blots were probed with NOS III antibody (Santa Cruz) at 1.5:1000 dilution. Lane assignments in both panels A and B are: (1) 0 hr, (2) 24 hr, (3) 48 hr, (4) 72 hr and (5) 96 hr post-angioplasty. Lane (6) represents the positive control, a lysate prepared from endothelium scraped from porcine coronary arteries. Protein loading was 20 μg/lane for panel A with the exception of lane 6 which contained 2 μg protein, while protein loading was 18.35 μg/lane for panel B with 4 μg protein in lane 6. This figure shows 2 distinct sample sets which are representative of 2 of 5 independent experiments.

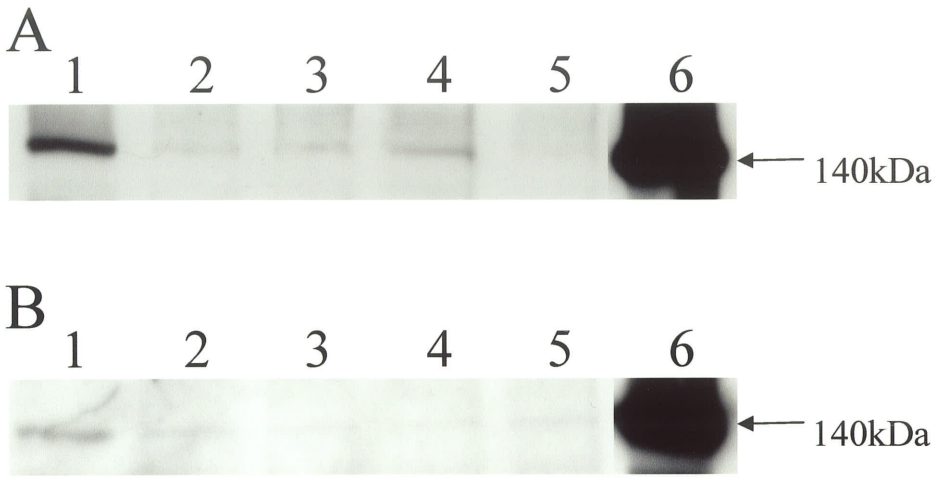

A
1
2
3
4
5
6
140kDa
B
1
2
3
4
5
6
140kDa

Figure 7: Comparison of eNOS Detection by Various Antibodies in Medial Preparations

Medial VSMC preparations were collected from denuded, 0 hour non-injured vessels as outlined in Material and Methods. The top panel was probed with polyclonal NOS III antibody (Santa Cruz) at a dilution of 1:1000 whereas the bottom panel was probed with monoclonal eNOS antibody (Transduction Laboratories) at a dilution of 1:2500. Both upper lanes contained 15 μg protein whereas the lower lanes contained 11 μg of protein. For both panels, lane 1 represents the medial preparation and lane 2, an endothelial cell sample. Similar results were obtained in three independent experiments.

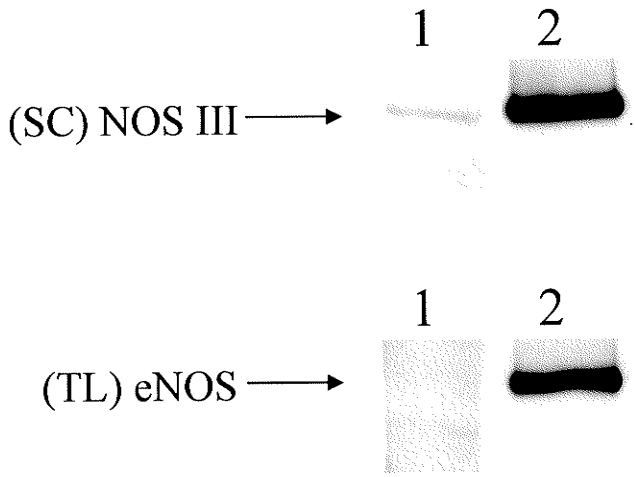
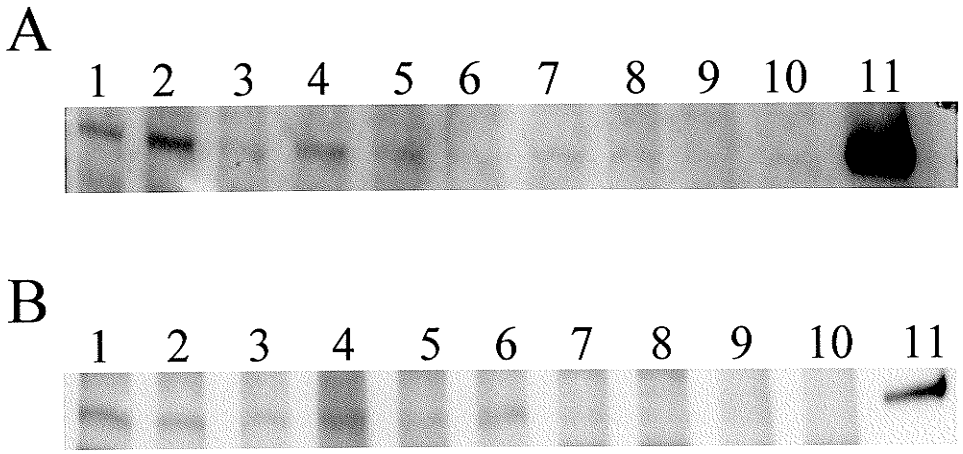

1
2
(SC) NOS III
1
2
(TL) eNOS

Figure 8: eNOS Expression in Non-Injured and Balloon-Injured VSMCs

Medial samples from non-injured (1,3,5,7,9) and balloon-injured (2,4,6,8,10) vessels were collected as outlined in the Methods section. The samples were run simultaneously to detect possible differences between the two conditions over time. Polyclonal NOS III (Santa Cruz) antibody was used at 1.5:1000 dilution (panel A) and monoclonal eNOS (Transduction Laboratories) antibody at 1:2500 dilution (panel B). Lanes for both panels A and B are representative of 0 hr (1,2), 24 hr (3,4), 48 hr (5,6), 72 hr (7,8) and 96 hr (9,10) samples. Lane 11 (A,B) represents the positive control, a lysate made from crude endothelium. Medial samples contained 20 μg protein per lane whereas lane 11 contained 2 μg endothelial cell lysate. These results are representative of 3 independent experiments.


A
1 2 3 4 5 6 7 8 9 10 11
B
1 2 3 4 5 6 7 8 9 10 11

compared to 3 minutes with the SC antibody to obtain a similar intensity for the eNOS band with the EC lysate positive control (Figure 7, lane 2).

4.2.1 Detection of eNOS in Normal and Injured Medial Preparations

To determine whether the tunica media of non-injured vessels also expresses eNOS, Western blot analysis was used to examine medial preparations of both non- and balloon-injured vessels. Samples were prepared at 24 hour intervals as described in Materials and Methods, and the blots probed with antibodies from 2 sources. As seen in Figure 8, eNOS expression was highest prior to being placed into culture (panels A and B, lanes 1 and 2) then decreased over time until 72-96 hours when eNOS was no longer detectable (panels A and B, lanes 7-10). Similar results were seen whether the tissue was subjected to balloon-injury or not.

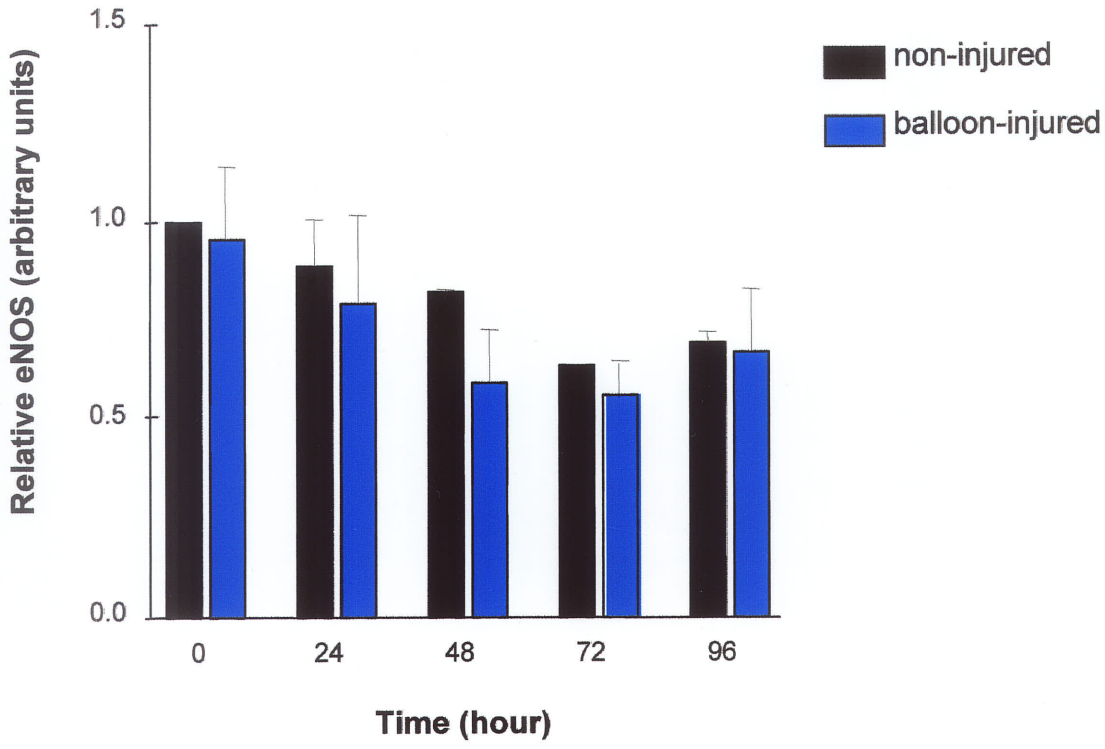
4.2.2 Analysis of Changes in Non- and Balloon-Injured eNOS Levels

To determine whether the variation in eNOS was representative of actual changes in eNOS levels in response to the experimental conditions employed, the Western blots were analyzed using two-way analysis of variance (ANOVA). The densitometry (OD) values obtained per blot were normalized to the 0 hour non-injured sample, set to an arbitrary unit of one. This was done to allow comparison between experiments while accounting for other independent factors (e.g. exposure time of autoradiography film for each experiment). Graphical representation of the results for 2-3 experiments employing the TL antibodies are shown in Figure 9. Based on the analysis, there were no statistically significant differences in eNOS expression over the time intervals of the

Figure 9: Time Course of eNOS Expression in Normal and Injured LADCAs

Medial VSMC preparations were extracted from uninjured coronary arteries as described in Materials and Methods. Densitometric data from 2-3 independent experiments were normalized, then analyzed by two-way analysis of variance (ANOVA). eNOS expression was detected by monoclonal anti-eNOS (TL) antibody at a dilution of 1:2500 comparing eNOS protein levels in medial samples at 24 hour intervals over 0 to 96 hours, in both normal and balloon-injured tissues. The data are plotted as means ± SEM with significant differences indicated (*, $p < 0.05$).

Western blot Analysis of Medial eNOS in LADCAs Post-Angioplasty



experiment ($p = 0.1249$). Analysis also suggests that the injury itself did not have a significant effect on eNOS expression ($p = 0.3201$). In addition, both normal (non-injury) and balloon-injury had similar effects at the different 24 hour intervals ($p = 0.9564$) indicating the interaction between injury and time were not significant.

To detect differences in eNOS expression in balloon-injured vessels over time, Western blots were analyzed using one-way ANOVA and Dunnett's Multiple Comparison test. Graphical representation of the results of three independent experiments using the SC antibody are shown in Figure 10. Statistically significant differences between the densitometry values of injured samples over 24 hour intervals were detected using one-way ANOVA ($p = 0.01$). Furthermore, Dunnett's post-test detected significant differences when comparing 0 versus 24 and 72 hours post-angioplasty ($p<0.05$), and when comparing 0 versus 48 and 96 hours, post-angioplasty ($p<0.001$).

4.2.3 Comparison of eNOS Levels in Medial VSMCs versus ECs

One-way ANOVA was used to compare eNOS levels in (0 hour) non-injured and balloon-injured medial VSMC, as well as EC samples (Figure 11). Western blot data was normalized to arbitrary units (AU) and is representative of 2-3 independent experiments. As shown in panel A, significant differences ($p<0.0075$) were found between the levels of EC eNOS compared with both normal and injured VSMC eNOS levels for the (SC) antibody. Dunnett's Multiple Comparison test was then used as significance was found overall between groups. Non-injured medial samples (with an AU of 1) were compared

Figure 10: Effect of Balloon-Injury on eNOS Expression Over Time

Medial VSMC preparations were extracted from balloon-injured coronary arteries as described in Materials and Methods. Densitometric data from 3 independent experiments were normalized, then analyzed by one-way analysis of variance (ANOVA) and Dunnett's Multiple comparison test. Expression was detected by polyclonal NOS III (SC) antibody at a dilution of 1.5:1000. eNOS protein levels in were examined in medial samples over 24 hour intervals from 0 to 96 hours. The data are plotted as means ± SEM with significant differences indicated (*, $p < 0.05$).

Western Blot Analysis of Medial NOS III in LADCA post-angioplasty

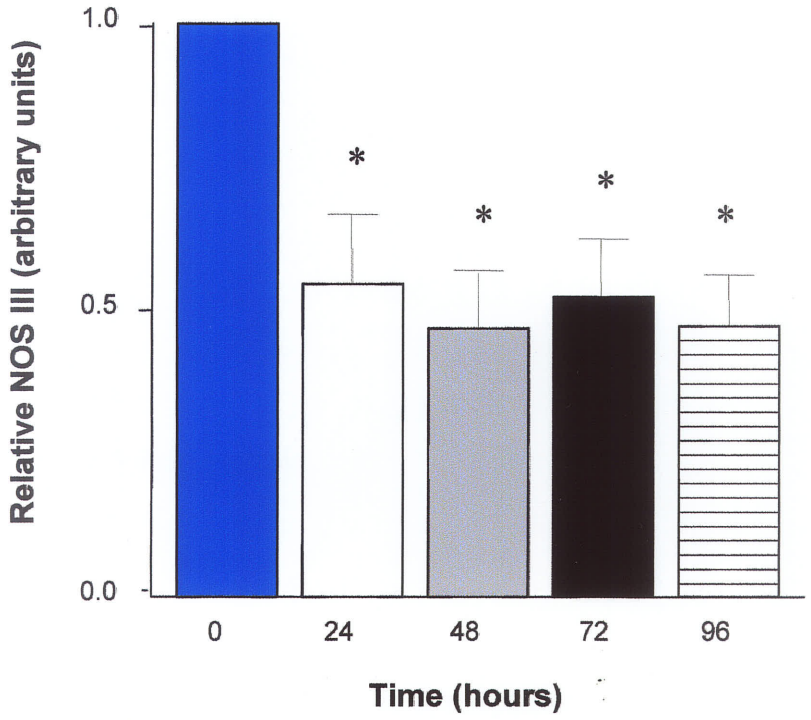
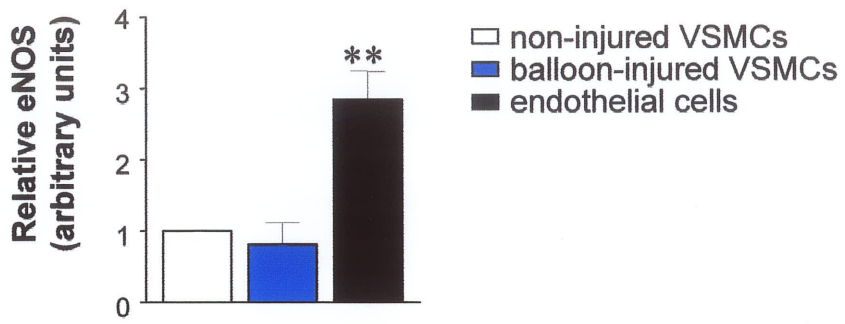
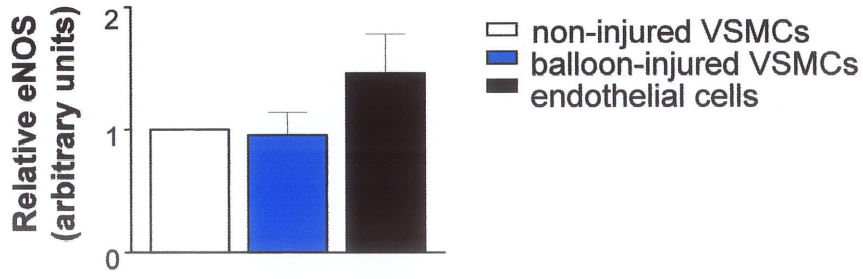


Figure 11: EC versus Medial VSMC NOS III
Comparison of eNOS expression in vascular ECs versus eNOS in the medial layer of normal and balloon-injured porcine LADCAs. Densitometric values from 2-3 independent experiments were normalized to arbitrary units (AU). Histograms in panels A and B compare levels of eNOS protein detected by (SC) and (TL) antibodies at a dilution of 1.5:1000 and 1:2500, respectively. The data are plotted as means ± SEM, with significant differences indicated (*, $p < 0.05$; **, $p < 0.001$).

A


Analysis of eNOS in ECs vs. Medial VSMCs with (SC) Ab
Relative eNOS (arbitrary units)
0
1
2
3
4
**
non-injured VSMCs
balloon-injured VSMCs
endothelial cells

B


Analysis of eNOS in ECs vs. Medial VSMCs with (TL) Ab
Relative eNOS (arbitrary units)
0
1
2
non-injured VSMCs
balloon-injured VSMCs
endothelial cells

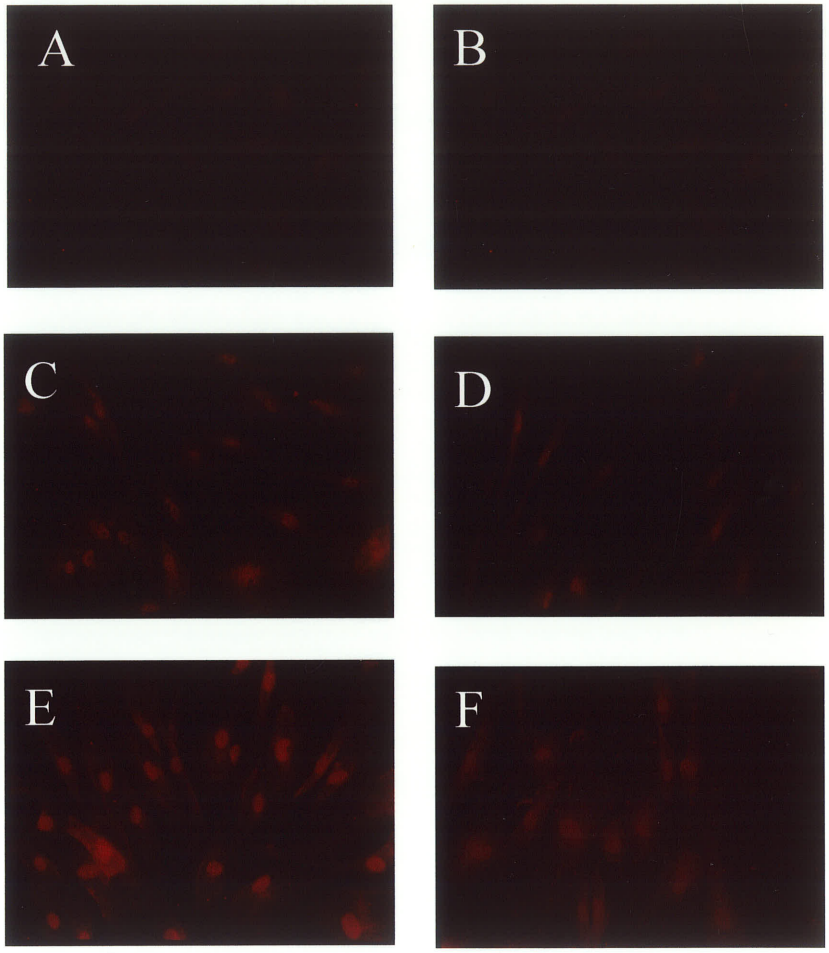
to balloon-injured VSMCs which had no significance ($p>0.05$), however, non-injured versus EC samples had significant differences in eNOS levels ($p<0.001$). In contrast to the (SC) antibody, based on the data also representative of 2-3 separate experiments, the (TL) antibody did not detect significant differences in eNOS expression between ECs and the non- and injured medial samples ($p = 0.2128$) (panel B). Therefore, comparisons made between the 0 hour non-injured and balloon-injured values show there was no significant difference in eNOS expression between these two conditions, for either the TL or the SC antibody ($p>0.05$).

4.3 eNOS Expression in Cultured VSMCs Upon Stimulation

Injury inflicted on the vessel wall upon inflation of the balloon-catheter is thought to induce or alter production of various agents such as Ang II post-injury (Pratt and Dzau, 1996). This may be relevant to the current study, since the literature indicates Ang II can affect NO/NOS expression and activity. The individual effects of Ang II or serum (consisting of many undefined substances) on VSMCs were therefore examined in cell culture. Porcine LADCA explants were used to prepare primary cell cultures following the procedures outlined in Materials and Methods. VSMCs were stained with polyclonal anti-ECNOS antibody (TL) in the presence or absence of mitogen stimulation (Figure 12). Quiescent VSMCs were stimulated with either 3% fetal bovine serum or 10^{-5} M Ang II. Although eNOS was not detectable by immunocytochemistry in unstimulated quiescent cells, both agents increased eNOS at 36 hours (panel C, D) and 72 hours (panel E, F) post-stimulation. The subcellular location of eNOS is difficult to determine without confocal studies or counterstaining with 4′,6-diamindino-2-phenylindole dihydrochloride

Figure 12: eNOS Localization Following Serum and Angiotensin II Stimulation of Cultured VSMCs

Quiescent VSMCs (A,B) were stimulated with 3% FBS (C,E) and 10^{-5} M Angiotensin II (D,F) for 36 (C,D) and 72 (E,F) hours, respectively. The cells were subsequently stained with polyclonal ECNOS antibody (Transduction Laboratories) at 1:100 dilution. Magnification: 250×


A
B
C
D
E
F

(DAP1), a cell permeable fluorescent probe for DNA. However, several reports have suggested eNOS is localized to the perinuclear Golgi complex both in a subconfluent bovine (bEnd.3) EC line and in primary bovine aortic ECs (Sessa et al., 1995; Govers et al., 2002a and b). Attempts to repeat this study failed, therefore Western blotting was utilized as an alternative to determine whether or not eNOS responded to Ang II and serum in cultured VSMCs.

4.3.1 Stimulated eNOS Expression Unaltered Over Time

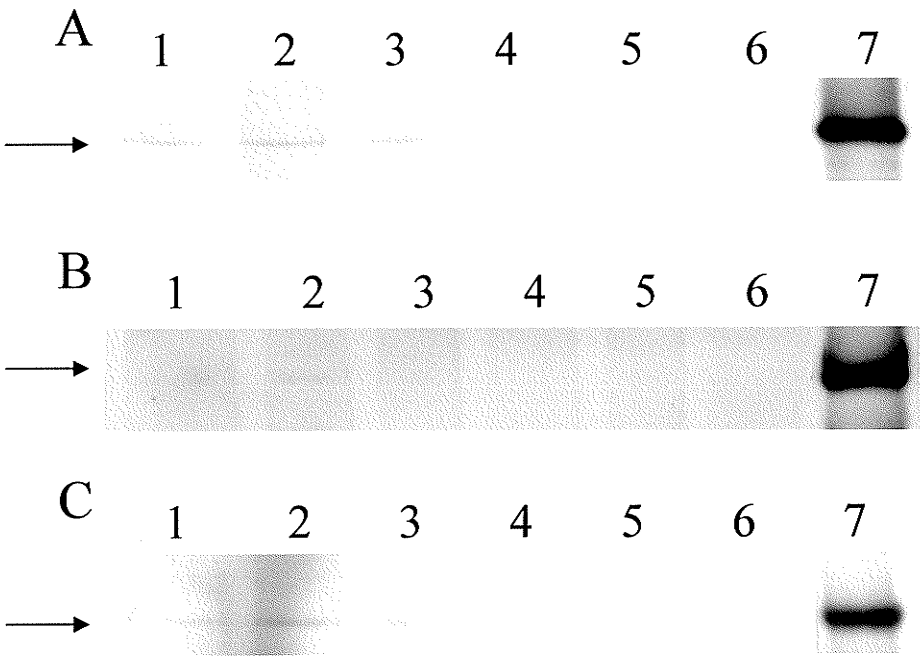
Western blot analysis using polyclonal NOS III (SC) antibody was used to determine whether eNOS was present in quiescent or stimulated VSMCs (Figure 13). Quiescent SMCs were treated with 1% FBS (panel A) or 10^{-6} M Ang II (panel B), then examined every 24 hours over a 96 hour period. eNOS was detected in quiescent, unstimulated VSMCs (lane 1) and at 2 and 24 hours (lanes 2,3), but was undetectable after 48 hours (lane 4) post-stimulation with serum. The lack of detectable changes in eNOS expression upon stimulation with Ang II may be due to reduced protein loading as shown in panel B, however, Ang II stimulation did not have a consistent effect on eNOS expression as determined in three independent experiments.

4.3.2 Effect of Varying Concentrations of Ang II on eNOS Expression

To determine if Ang II had a concentration-dependent as opposed to a time-dependent effect on eNOS expression, VSMCs were examined for eNOS at 48 hours after stimulation with varying concentrations of Ang II (10^{-9}-10^{-5} M) (Figure 13, panel C). Unstimulated VSMCs exhibit eNOS expression (lane 1). eNOS levels remained

Figure 13: Effect of Angiotensin II and Serum Stimulation on eNOS Expression

In panels A and B, quiescent VSMCs were stimulated with 1% FBS and 10^{-6} M Ang II, respectively, for 2 (lane 2), 24 (lane 3), 48 (lane 4), 72 (lane 5), and 96 (lane 6) hours. In panel C, quiescent VSMCs were stimulated for 48 hours with varying concentrations of Ang II: 10^{-9} M (lane 2), 10^{-8} M (lane 3), 10^{-7} M (lane 4), 10^{-6} M (lane 5) and 10^{-5} M (lane 6). For all panels, unstimulated control SMC samples are shown in lane 1, while positive controls (Transduction Laboratories) are shown in lane 7. All blots were probed with polyclonal NOS III antibody (Santa Cruz) at 1.5:1000 dilution. The protein concentrations loaded per lane in panels A-C are: 15.52, 6.40 and 14.42 μg for the SMC samples and 3, 3 and 4 μg protein for the EC positive control (lane 7). The arrows indicate the 140 kDa eNOS protein. Panels B and C are examples of the results obtained from one of three and four independent experiments, respectively.


A
1
2
3
4
5
6
7
B
1
2
3
4
5
6
7
C
1
2
3
4
5
6
7

detectable with both 10^{-9} and 10^{-8} M Ang II (lane 2 and 3) but decreased with higher concentrations of Ang II. Of note, these results were inconsistent in repeated experiments and the effects of Ang II failed to be inhibited by the Ang II receptor antagonists losartan and PD123319 (data not shown).

4.4 eNOS Gene Expression in Both Normal and Injured PCAs

To confirm the finding that eNOS was expressed in the medial layer, reverse-transcription polymerase chain reaction (RT-PCR) was performed to measure eNOS mRNA levels. Relevant primers for porcine eNOS, smooth muscle myosin heavy chain (MHC), von Willebrand factor (vWF) and iNOS were designed as described in Methods.

eNOS mRNA was present in both non-injured and balloon-injured medial samples prior to culture (Figure 14, lanes 2 and 6) and in all samples prepared from cultured vessels (lanes 3-5 and 7-9). As was seen with the Western blot analysis, the levels of eNOS mRNA in the EC positive control sample (lane 10) were much greater than those found in the medial samples. The presence of eNOS and von Willebrand factor (lanes 11 and 12) was used as positive markers of ECs. Consequently, the lack of vWF in a representative medial sample (lane 11) indicated the medial preparations were free of endothelial cells. To further confirm the preparations were free of contaminating ECs, the samples shown in Figure 14 were tested by RT-PCR for both vWF and MHC (Figure 15). vWF mRNA was only amplified in the EC sample (lane 13), whereas MHC mRNA was observed in a representative medial VSMC sample but not the EC sample (lanes 3 and 4). These data support the evidence that eNOS can be expressed by VSMCs, but reproducibility

Figure 14: Comparison of eNOS Gene Expression in Balloon- versus Non-Injured LADCAs Over Time

RT-PCR amplification was performed on RNA (2 μg) extracted from non-injured (lanes 2-5, 11) and balloon-injured (lanes 6-9) porcine coronary arteries as described in Materials and Methods. Total RNA from medial preparations was extracted after: 0 hr (2,6), 24 hr (3,7), 48 hr (4,8) and 72 hrs (5,9) of culture. The eNOS mRNA yields a band of 364 bp. The positive control was RNA from crude ECs (lane 10). vWF primers (289 bp product) were tested with both non-injured (0 hour) SMC (lane 11) and EC RNA (lane 12). DNA molecular marker VIII (Roche) is shown in lane 1.

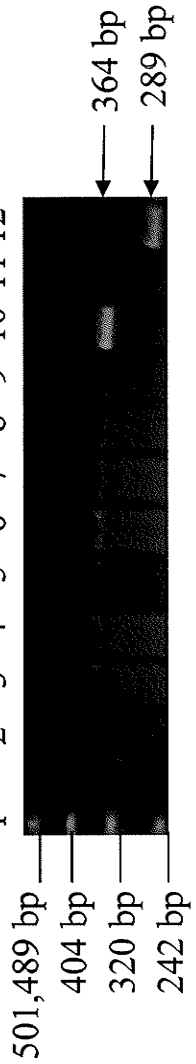
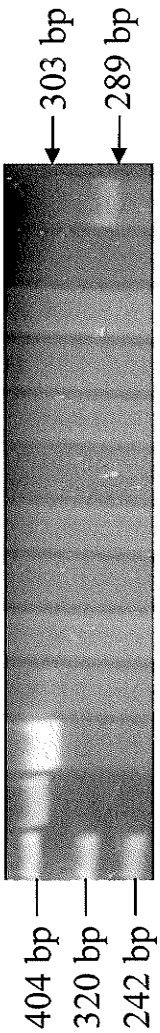

1 2 3 4 5 6 7 8 9 10 11 12
364 bp
289 bp
501,489 bp
404 bp
320 bp
242 bp

Figure 15: vWF and SM Myosin Heavy Chain Gene Expression Post-Angioplasty

RT-PCR amplification was performed on total RNA (2 μg) extracted from non-injured (lanes 3, 5-8) and balloon-injured (lanes 9-12) coronary artery medial preparations and EC samples (4, 13). RNA was extracted from medial samples at: 0 hr (lanes 3,5,9), 24 hr (lanes 6, 10), 48 hr (lanes 7, 11) and 72 hr (lanes 8,12), whereas EC RNA was extracted from endothelium removed from fresh LADCA. A positive control for the PCR reaction (shown in lane 2) is represented by 323 bp product (control RNA and primers provided by Promega). Expression of von Willebrand factor (vWF) (lanes 5-13) and smooth muscle myosin heavy chain (lanes 3 and 4) was also assessed (289 and 303 bp products, respectively). DNA molecular marker VIII (Roche) is shown in lane 1.


1 2 3 4 5 6 7 8 9 10 11 12 13
303 bp
289 bp
404 bp
320 bp
242 bp

among specific time points and/or in comparing the effects of injury, was problematic. For instance, in a separate experiment, eNOS was detectable in the non-injured samples but not in the balloon-injured samples (Figure 16).

The concentration of RNA within the medial samples was calculated using UV/Visible spectrometry as described in the Methods (section 3.2.5.2) and loaded accordingly for 1-2 μg per RT-PCR sample reaction. To confirm equal RNA loading, primers for an internal control (GAPDH) were initially selected to be run with the different medial sample conditions. However, the optimal number of cycles for GAPDH is approximately 20-25 cycles, whereas for eNOS 30-35 cycles were necessary for detection of medial eNOS mRNA. A GAPDH control was therefore not incorporated in the current studies. Consequently, only the presence or absence of eNOS mRNA in the tunica media was determined, and differences between time points or the effects of injury versus non-injury were not compared. Nevertheless, it is evident from these results that eNOS is expressed by the VSMCs within arteries, albeit at significantly reduced levels compared to ECs.

To ensure that the eNOS primers were amplifying the appropriate sequence, the eNOS RT-PCR products from ECs and control non-injured medial VSMCs were cloned and sequenced. The sequence of the amplified PCR product confirmed that the porcine primers designed were indeed specific for *Sus scrofa* eNOS. This sequence was compared with the "BLAST-N (nucleotide)" program, which searches the GENBank database for other proteins/genes with a corresponding sequence. Sequences producing significant alignments for the cloned RT-PCR product are shown in Figure 17.


A B C D E F G H I
364 bp
404 bp
320 bp

A

Sequences producing significant alignments:	Score (bits)	E Value
gi|1762433|gb|U59924.1|SSU59924 Sus scrofa nitric oxide syn...	694	0.0
gi|15077875|gb|AF400594.1|AF400594 Homo sapiens endothelial...	543	e-152
gi|189211|gb|M93718.1|HUMNIOXSYN Human nitric oxide synthas...	543	e-152
gi|10835160|ref|NM_000603.1| Homo sapiens nitric oxide synt...	535	e-149
gi|434699|gb|L26914.1|HUMNOSA Human nitric oxide synthase m...	535	e-149
gi|189259|gb|M95296.1|HUMNOS Human nitric oxide synthase mR...	535	e-149
gi|163421|gb|M99057.1|BOVNIOXSY Bovine nitric oxide synthas...	488	e-135
gi|162976|gb|M89952.1|BOVECNOS Bos taurus endothelial nitri...	488	e-135
gi|6980033|gb|AF223471.1| Ovis aries endothelial nitric oxi...	472	e-130
gi|4769080|gb|AF146041.1|AF146041 Cavia porcellus clone 2 e...	472	e-130
gi|4769078|gb|AF146040.1|AF146040 Cavia porcellus clone 1 e...	472	e-130
gi|163426|gb|M95674.1|BOVNOS Bos taurus nitric oxide syntha...	464	e-128
gi|5814291|gb|AF143503.1|AF143503 Canis familiaris nitric o...	410	e-112
gi|6679089|ref|NM_008713.1| Mus musculus nitric oxide synth...	375	e-101
gi|26346806|dbj|AK077896.1| Mus musculus 13 days embryo mal...	375	e-101
gi|1518955|gb|U53142.1|MMU53142 Mus musculus endothelial co...	375	e-101
gi|1144486|gb|U33832.1|SSU33832 Sus scrofa nitric oxide syn...	355	3e-95
gi|3676237|emb|AJ011116.1|RNO011116 Rattus norvegicus mRNA ...	353	1e-94
gi|7259221|emb|AJ249546.1|RNO249546 Rattus norvegicus parti...	293	1e-76
gi|21686529|gb|AF519768.1| Homo sapiens nitric oxide syntha...	220	1e-54

B

Sus scrofa nitric oxide synthase (NOS) mRNA, complete cds
gi|1762433|gb|U59924.1|SSU59924
Length = 4024 Score = 694 bits (350),
Expect = 0.0 Identities = 353/354 (99%) Strand = Plus / Plus

```
Query: 7    cctgcactatggagtctgctccacgtggctgagccagctcaagcctggagaccctgtacc
             ||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct: 2922 cctgcactatggagtctgctccacgtggctgggccagctcaagcctggagaccctgtacc

Query: 67   ctgcttcatcagggcggctccctccttccggctgccacctgatcccagcttgccctgcat
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 2982 ctgcttcatcagggcggctccctccttccggctgccacctgatcccagcttgccctgcat

Query: 127  cctggtgggcccaggcacaggcattgccccttccggggattctggcaggagcggctgca
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3042 cctggtgggcccaggcacaggcattgccccttccggggattctggcaggagcggctgca

Query: 187  tgacattgagagcaaagggctgcagcccgcccccatgaccttggtgttcggctgccgatg
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3102 tgacattgagagcaaagggctgcagcccgcccccatgaccttggtgttcggctgccgatg

Query: 247  ctcccagctcgaccatctctaccgcgacgaggtgcaggatgcccagcagcgcggggtgtt
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3162 ctcccagctcgaccatctctaccgcgacgaggtgcaggatgcccagcagcgcggggtgtt

Query: 307  tggacgcgtcctcaccgccttctcccgggaacccgacagccctaagacctacgt 360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 3222 tggacgcgtcctcaccgccttctcccgggaacccgacagccctaagacctacgt 3275
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4.5 Activity of Cultured VSMC eNOS

Activity of the eNOS detected in medial VSMCs could not be determined as the methods employed, both colorimetric and radioactive, were unable to detect either nitrite and nitrate, or citrulline production, respectively. These negative results apply to both samples and positive controls.

5. DISCUSSION

Clinically, percutaneous transluminal coronary angioplasty is an effective intervention for re-establishing blood flow in coronary arteries. However, its major drawback is restenosis which exhibits an incidence of 30-50% in patients within 6-12 months (Hinohara, 2001; Nobuyoshi et al., 1988). Restenosis is presumed to result from the insult generated on the vascular tissues during PTCA, since inflation of the balloon catheter has many damaging effects. Among the most severe events is denudation of the vessel luminal wall. Since the endothelium is an essential component of the vasculature that regulates vasomotor tone, thrombogenicity and synthesis of various autocrine/paracrine agents, its removal (or dysfunction) may underlie the pathological effects that lead to restenosis. The loss of eNOS, the major isoform of nitric oxide synthase found in the endothelium, may be of particular significance since nitric oxide can modulate basal tone, inhibit SMC proliferation and migration, and reduce platelet adherence, all factors that contribute to restenosis.

The novel finding of these studies is the demonstration that eNOS is expressed by the vascular smooth muscle cells of porcine LADCAs. Three distinct methods were used to confirm this observation. Initially, we examined the effect of balloon-injury on the expression of eNOS using immunohistochemistry. eNOS levels decreased following denudation of the artery, but eNOS was still detectable within the tunica media of the coronary arteries (Figure 2 and 4). In addition, Western blot analysis of cell lysates from pooled medial VSMC preparations confirmed the immunohistochemistry findings. Western blotting detected a 140 kDa protein in these medial preparations, as well as in

the EC controls. eNOS expression appears highest within the balloon-injured samples prior to culture (at 0 hours) as seen in Figure 6 (panels A and B, lane 1), and statistically significant differences ($p = 0.01$) were found when using one-way ANOVA to compare eNOS expression post-angioplasty over 24 hour intervals with the SC antibody (Figure 10). Dunnett's Multiple Comparison test identified significant differences between the uncultured 0 hour balloon-injured medial samples with all other time points (24 to 96 hours), but not among the other time intervals of injured samples. This suggests that the largest change in eNOS levels occur with the first 24 hours. However, it was also noted that eNOS was expressed in medial preparations from normal, non-balloon injured porcine LADCAs (Figure 8). Furthermore, like the balloon-injured samples, eNOS expression in non-injured preparations rapidly declined in culture. To quantify the changes in eNOS expression, the band intensities obtained in 2-3 independent experiments were measured by densitometry and subsequently normalized for each experiment. Using 2-way ANOVA, the differences found in eNOS expression over time, detected with the TL antibody, were not statistically significant ($p>0.05$) for either normal or injured samples (Figure 9). These data would suggest that eNOS levels within the tunica media may be constitutive and that balloon-injury does not alter eNOS expression within the medial layer. This also suggests that placing the medial samples in culture may have an impact on eNOS protein, not associated with the effects of the injury itself.

As mentioned in Methods (section 3.2.7), both parametric (one- and two-way ANOVA) and non-parametric (Kruskal-Wallis) statistical tests were performed. Kruskal-Wallis is a

non-parametric test that parallels the two-way ANOVA and is used when data is not normally distributed. This test is inherently less powerful as it disregards some information when original values are compared in rank and risk missing real but subtle differences. Kruskal-Wallis is often followed by Mann-Whitney test which parallels the parametric t-test and is used to find differences between groups, only if significant differences are found overall. Similarly, Dunnett's or Bonferroni Multiple Comparison tests are used as a follow-up test if significant differences are detected between variables e.g. over time, with injury, using ANOVA. No significant difference in eNOS expression was detected between non-injured and balloon-injured medial samples with ANOVA (Figure 9) so the Dunnett's post-test was not employed.

One problem associated with the small number of samples used in our studies is the difficulty in determining or inferring the distribution, whether Gaussian or not, of an entire (sample) population. With a small sample size ($n < 12$), a non-parametric test has limited applicability since "the p value tends to be high and the test lacks statistical power... making it more difficult to detect real differences as being statistically significant" (GraphPad Software, Inc. user manual (1999)). With very small groups like those in our studies ($n = 2$ or 3), non-parametric tests such as Kruskal-Wallis have zero power as the confidence interval will always be less than 95% ($p > 0.05$) no matter how the groups differ (GraphPad Software, Inc. user manual (1999)). On the other hand, the risk with parametric tests is that the p value may be inaccurate if the distribution is not Gaussian especially if the sample size is small (e.g. $n < 12$). This type of test assumes that equal variance exists across the sample groups and if this is not the case, a non-

parametric test should be chosen. However, a dilemma exists as the Kruskal-Wallis test will always give a p value greater than 0.05 if the total sample size is seven or less (GraphPad Software, Inc. user manual (1999)). Based on these considerations, one- and two-way ANOVA were chosen for making our statistical comparisons, keeping in mind the limitations of significance of the result.

It may be appropriate to discount the significance found in injured medial samples over time (Figure 10) as it does not include non-injured samples in its analysis and is therefore of less meaning (decreased power of test). It is inaccurate to do a separate t test for each pair of groups if data is collected from more than two groups. Instead, all groups must be compared at once to allow for a more powerful and meaningful result (Motulsky, 1995). Analysis of the combined findings of both injured and non-injured tissues suggest that eNOS levels are maintained and this evidence strengthens the argument that injury itself does not alter eNOS expression.

As mentioned previously, vascular shear stress, a hemodynamic force exerted on the surface of the endothelium by the flow of blood, is one stimulus capable of increasing the expression of eNOS (Sessa et al., 1994; Nishida et al., 1992). Different types of forces such as unidirectional shear stress act directly upon the ECs, whereas transmural pressure acts outward upon the vessel walls to affect both EC and SMCs (Ballermann et al., 1998). As inflation of the balloon-catheter also exerts an outward pressure, it can be speculated that this effect may contribute to eNOS expression in the medial VSMC layer. Balloon-angioplasty may decrease EC eNOS by causing damage to the endothelium, however, our

findings suggest that balloon-injury does not have a significant effect on medial eNOS expression *in vitro* when compared to the non-injured medial eNOS levels (Figure 11, panels A and B). The tendency for VSMC eNOS expression to rapidly decline in culture in both non- and injured samples may only be an *in vitro* phenomenon due to the lack of flow within organ culture (Figure 8) and, therefore, a consequence or limitation of this model. In the normal vasculature, the constant blood flow and the resultant shear stress maintain higher levels of expression in the ECs. In addition, the presence of growth factors or other substances in the culture medium may have an influence on eNOS expression by medial VSMCs unlike that found *in vivo*.

To determine whether cultured medial VSMCs express eNOS, VSMCs obtained from free-floating porcine LADCA explants were examined. In addition, as Ang II is one agent released following balloon-injury (Adams et al., 2000; Schwartz et al., 1996) and is associated with an elevation in expression of the constitutive NOS (Olson et al., 1997), the effects of Ang II and fetal bovine serum were studied. As outlined in Results, immunohistochemical analysis showed eNOS was elevated at 36 and 72 hours post-stimulation (Figure 12), although the peri-nuclear localization found in ECs by Sessa et al. (1995) and Govers et al. (2002a,b) was not determined in our studies. Since the result of this single experiment could not be reproduced, Western blotting was employed to verify that eNOS was indeed present in stimulated cultured VSMCs. This approach revealed eNOS was expressed in VSMCs before and after stimulation with 1% FBS. Of note, there was an apparent decline in eNOS after 48 hours of stimulation (Figure 13A, lane 4). However, 10^{-6} M Ang II stimulation did not have a consistent effect on eNOS

expression over the experimental time period (panel B) as displayed by one result over 3 independent experiments.

The influence of various concentrations of Ang II at 48 hours was also tested. This experiment (Figure 13C) suggested that high levels of Ang II (10^{-5} M) caused a decline in eNOS, however, this result was inconsistent over four separate experiments. As a result, Ang II was not examined further. The ineffectiveness of the Ang II antagonists on eNOS expression (data not shown), tested in 3 independent experiments, further suggests that Ang II does not play a significant role in eNOS expression in cultured VSMCs. Nevertheless, it is possible that the time at which the Ang II concentration curve was examined in this study (48 hr) may have missed the points where a detectable change had occurred.

Lastly, the presence of eNOS protein in medial VSMCs implies that eNOS mRNA must be produced within these cells. To verify this supposition, the technique of reverse transcription polymerase chain reaction (RT-PCR) was employed. eNOS mRNA was detected with this method in both non-injured and balloon-injured medial samples (Figure 14). Cloning and sequencing of the amplified RT-PCR product confirmed that the eNOS primers specifically amplified eNOS mRNA (Figure 17).

It may be speculated that expression of eNOS post-angioplasty is due to regeneration of the endothelium. However, the conditions of our *in vitro* organ culture model do not allow for re-endothelialization (Werner and Zahradka, unpublished results), as re-

generation of the endothelium requires migration of healthy ECs from proximal and distal regions. It has also been noted by DiCorleto et al. (1979) that the ability to regenerate ECs after balloon-catheter injury is limited in many animal species. To ensure that ECs were not contaminating the medial preparations, RT-PCR amplification of von Willebrand factor (vWF), a marker often used for identifying endothelium (Sessa et al., 1994; Nakamura et al., 1997), and smooth muscle myosin heavy chain mRNA were used to distinguish between these cell types. The vWF was only detected in EC samples (Figure 14, lane 12 and Figure 15, lane 13), whereas SM myosin heavy chain was only detected in VSMC samples (Figure 15, lane 3). Thus, the presence of eNOS could only be explained by its expression in VSMCs.

The greatest difficulty encountered during this investigation was the variability in eNOS staining with immunohistochemistry. There was non-uniform staining within different regions of the medial section and variability in eNOS expression between different porcine vessels which had been maintained in the same conditions, at identical time points. For example, Figure 2 displays IH staining within the medial layer with a monoclonal anti-eNOS antibody (Calbiochem) at 48 hours (panel C). In contrast, there was no medial staining seen with the polyclonal anti-NOS III antibody (SC) at 48 hours (Figure 3-5, panels G, C and E). Furthermore, experiments presented in Figure 4 show staining in some samples at 53 and 72 hours post-angioplasty, but not in others since eNOS was not detected at the latter time point in Figure 5 (panel G). Similar to immunohistochemistry, the results obtained by Western blot analysis reflect the variability in eNOS expression seen in both (non- and balloon-injured) 0 hour controls.

This may be due to i) intrinsic variability within our organ model especially with respect to consistency in producing the injury, ii) inherent differences between individual pigs in their response to injury, and iii) the sensitivity of the detection method (immunohistochemistry and Western blotting). Different antibodies detect different epitopes, which may be masked or exposed depending on the technique being applied. Western blotting tends to expose all epitopes, however, denaturation by SDS may alter epitopes depending on the structural integrity of the native eNOS protein. On the other hand, fixation of tissues for immunostaining does not typically denature the sample proteins to the same degree as during Western blotting, so retention of the native configuration may mask epitopes otherwise detected by the same antibody. Nevertheless, the limited number of samples used per time point (2-3), per experiment may have also increased the potential for false negatives (type II or β-type errors) (Muller et al., 1992).

The differences observed between the immunohistochemical and Western blotting methods may reflect the conditions mentioned above, as well as a low level of eNOS expression in medial VSMCs compared to ECs (Figure 11, panel A). The ability of the antibodies to detect the low levels of eNOS within the medial tissues may be one factor contributing to this discrepancy. A study by Teng et al. (1998) also attributed the inability to detect eNOS protein to low expression in rabbit gastric or human intestinal SMCs. It must be noted that all Western blots were loaded with moderate amounts of protein (under or equal to 20 μg per lane), whereas 35-80 μg protein per lane has been reported in the literature (Hendrickson et al., 1999; Comini et al., 1996; Liao et al., 1995; Tidball et al., 1998). These differences may contribute to the variable detection seen for

eNOS in the medial samples for this study. The lack of a significant difference ($p = 0.2128$) in EC and medial VSMC eNOS levels obtained with the TL antibody (Figure 11, panel B) supports this interpretation. However, the presence of eNOS within the VSMC of non-injured vessels was also demonstrated by RT-PCR, thus confirming that this finding is not an artifact contingent on antibody specificity.

Despite the variability in the pattern of eNOS expression, we have established that eNOS is present within VSMCs of porcine LADCAs. This finding, although new, must be interpreted with caution. Presently, many researchers have failed to detect the presence of eNOS protein in medial VSMCs (Brophy et al., 2000; Sanders et al., 2000; Fukuchi and Giaid, 1999; Picard et al., 1998; Wilcox et al., 1997; Sessa et al., 1992). Why were we then able to detect SMC eNOS? It is plausible that the experimental system provided the necessary advantage for this purpose. For example, our *in vitro* organ culture does not allow re-endothelialization, post-angioplasty (Werner and Zahradka, unpublished results). The absence of ECs makes the much lower eNOS levels of VMSCs easier to detect as can be seen when comparing the denuded and non-denuded vessel segments (Figure 2 and 4) with an immunohistochemical approach. Another plausible factor for our findings may be the method of extraction and/or our use of medial preparations. eNOS is known to be constitutively expressed, typically within ECs and can be induced by various stimuli. Extracting the medial preparation includes gently denuding the endothelium with a scalpel blade followed by repeated flushing with ice-cold buffer. This should remove all ECs (as demonstrated by RT-PCR for vWF), however, as the medial layers are separated from the underlying adventitial layer, an effect not unlike

injury may be produced. Although the non- and balloon-injured vessels were handled identically, the fact that all tissue samples are stimulated to some degree during their isolation makes it appropriate to refer to them as non-balloon injured and balloon-injured medial samples. It is also well known that the medial SMC population is heterogeneous with respect to their growth and differentiation properties. The use of medial tissue preparations therefore avoids the isolation of uniform subpopulation(s) of migratory VSMCs from explanted LADCAs (Saward and Zahradka, 1997a) or cells altered by enzymatic digestion. Although a culture model does not reproduce the *in vivo* environment (e.g. pulsatile flow producing shear stress, hemodynamic substance or infiltrating cell interaction), by utilizing both medial preparations and organ culture, it is nevertheless possible to look at total VSMC populations, uniform/pure VSMC response in isolation from the adventitia, the vasa vasorum and associated nerves, as well as the native structure of the vessel itself.

The presence of eNOS in VSMCs was shown by immunohistochemistry, Western blotting and RT-PCR. Investigators such as Picard et al. (1998) have speculated that cells other than vascular endothelium may begin to express eNOS in the arterial wall post-injury. Furthermore, Comini et al. (1996) have found that in rats with CHF there was a shift in the location of eNOS protein expression from aortic ECs to VSMCs. Although the mechanism of eNOS up-regulation was not identified, they proposed that the increased expression in SMCs was a compensatory mechanism for severe decreases in eNOS in the endothelium. In agreement with our findings, they also found eNOS in the SMC layer of control rats (Comini et al., 1996). These observations, however, differ

from other reports which have suggested that the decrease in eNOS resulting from balloon-injury denudation can be compensated for by increased iNOS expression in medial and neointimal VSMCs (Groves et al., 1986; Groves et al., 1995). While expression of iNOS can be induced in VSMCs (Koide et al., 1994; Scott-Burden et al., 1997) by various cytokines/inflammatory agents, an increase in nNOS and iNOS protein levels was not detected by Western blotting in our medial preparations (data not shown) even after prolonged exposure of the autoradiography film. In addition, whereas eNOS mRNA has been detected in ECs of normal vessels, iNOS and nNOS mRNA have not been observed in the intimal or medial layers (Wilcox et al., 1997). In our RT-PCR experiments, iNOS mRNA was also not detected in the medial VSMC preparations (data not shown).

Attempts to measure the activity of medial VSMC eNOS were unsuccessful. Colorimetric activity assays utilizing Greiss reagents to indirectly detect NO production through its breakdown products, nitrite and nitrate, were tested initially. The background readings were high even when phenol red was left out of the media. This observation may indicate that the nitrate /nitrite levels are elevated in the media used for culture, thus limiting the sensitivity of the assay. A second NOS assay that measures NO through the conversion of [^{3}H] L-arginine to L-citrulline, a by-product of NO synthesis, was then employed. This indirect approach bypasses the need for specialized equipment to detect NO prior to its rapid decay (half-life of 3-5 seconds). However, despite following instructions and purifying the radioactive substrate, [^{3}H] L-arginine, background counts obtained solely with the substrate were sufficiently high that they interfered with the

accuracy of the test, for all VSMC samples as well as the positive control (EC).

What is the future for vascular eNOS research? Nitric oxide synthases and eNOS gene transfer, in particular, have been shown to be effective tools for inhibiting both VSMC proliferation and migration (Sato et al., 2000; Gurjar et al., 1999; Sharma et al., 1999; Janssens et al., 1998). By over-expressing human or recombinant forms of eNOS in rat and porcine VSMCs, it has been possible to increase local NO production within the vascular wall (Kullo et al., 1997). As mentioned previously, VSMC proliferation and migration are key elements in the development of restenosis. Studies by Varenne et al. (1998) and Janssens et al. (1998) have demonstrated that adenoviral-mediated transfer and over-expression of human eNOS in the medial and adventitial cells reduced luminal narrowing following coronary angioplasty due to NO's effects on neointimal proliferation and vascular remodeling. Increased levels of the tissue inhibitor of matrix metalloproteinases 2 (TIMP-2) and decreased matrix metalloproteinases (MMP-2 and -9) have also been associated with increased NO production (Gurjar et al., 1999). In addition, Sato et al. (2000) have found that eNOS gene transfer inhibits cell proliferation by delaying cell cycle progression by up-regulating $p27^{KIP}$ and $p21^{CIP}$. Shears et al. (1998) have found that the adenoviral-transfer and transient over-expression of iNOS can effectively reduce intimal hyperplasia if initiated at the time of vascular injury but cannot induce regression of previously established neointimal lesions.

Although eNOS exhibits the broad capabilities necessary for developing a gene therapy approach for controlling restenosis, similar limitations may exist. Our study indicates

that transfer of the eNOS gene into medial SMCs should lead to expression as VSMCs can express eNOS, corresponding to studies by Kullo et al. (1997) which found that adenoviral gene transfer of eNOS into porcine coronary SMCs produced a functional, active enzyme. Further comprehensive study of these issues is therefore warranted before clinical trials are considered.

6. CONCLUSION

Our studies provide evidence in support of the notion that eNOS is expressed within the normal tunica media of porcine LADCAs. This finding was confirmed by examining the levels of eNOS mRNA and protein within vascular tissues and isolated VSMCs. As well, our studies have shown that medial eNOS levels are at much lower levels than in ECs. However, the time course over which eNOS is expressed has not been conclusively identified. Therefore, further studies will be necessary to determine the exact timing and conditions that alter eNOS expression. Furthermore, eNOS gene expression in cultured VSMCs needs to be assessed in order to clarify whether its expression is modulated in response to changes in VSMC environment. It can be speculated that medial VSMC eNOS expression is constitutive as its expression is not significantly affected by balloon angioplasty (PTCA). An examination of the effects of Ang II and FBS on cultured VSMCs suggests that these agents also do not influence eNOS expression, since eNOS was detected in quiescent, unstimulated cells. Our findings must be interpreted with caution, however, as the natural individual variability in response of the porcine vessels appeared to add to the inconsistency of the changes detected in eNOS expression over time, as did the lack of strength in the statistical analysis due to small sample size. The absence of blood flow following placement into organ culture and consequence loss of shear stress stimulus may be the main reason for the possible decline in eNOS expression. It is worth noting however, that this trend was statistically insignificant. Caution must also be applied as these situations may not reflect the findings *in vivo* or the clinical setting, post-angioplasty.

Further studies will be necessary to clarify the findings reported in this thesis, and it would be most instructive to examine eNOS in an *in vivo* porcine model where normal pulsatile flow exists. In addition, eNOS could be studied in different cell cultures (explanted versus enzymatically-digested) and medial VSMCs from rat or rabbit vessels with antisense depletion experiments used to determine function. As mentioned previously, it is known that damage to vessels during angioplasty is not uniform. Consequently, eNOS expression may vary due to different degrees of injury. In addition, the heterogeneity of the medial VSMCs may further exacerbate this variability. Studies employing *in situ* hybridization may therefore assist in defining the expression and profile of the eNOS gene within the medial layer of porcine LADCAs before and after injury.

There is a need to determine whether eNOS is active in medial VSMCs and to determine the conditions (e.g. Km, turnover rate) that control its activity. Questions arise concerning the implications of eNOS activity within the medial layer. Similar to its actions within the EC, is it producing constitutive (basal) levels of NO and can it become uncoupled to produce damaging superoxide or peroxynitrite in pathological situations? Could its activity play a protective role in the disease process to help minimize dysfunction or can it contribute to further vessel pathology? All three NOS isoforms are reported to produce oxidants although the mechanisms under which it does so are still unclear. eNOS has been known to produce oxygen-derived free radicals under certain pathological conditions where there is a deficiency of tetrahydrobiopterin (BH_4) (Cosentino et al., 2001; Laursen et al., 2001; Cosentino and Luscher, 1998; Vasquez-Vivar et al., 1998; Xia

et al., 1998) or changes in GSH and superoxide dismutase (SOD) (Andrew and Mayer, 1999). In contrast, it has also been suggested that eNOS increases SOD expression in VSMCs to prevent superoxide-mediated degradation of NO (Fukai et al., 2000). Clearly, further studies are needed in order to examine these questions. As eNOS is playing an increasingly larger role in gene therapy for the purpose of inhibiting VSMC migration and neointimal proliferation, the investigation of normal eNOS expression, regulation and activity in vascular cells has only just begun.

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