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Vasoregulatory Gene Expression and Vascular Contractility Following Hypoxia

by

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DEDICATION

To Mom and Dad

With all My Love

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Michael E. Ward for providing me with an enriching experience throughout my doctoral studies. His commitment to the scientific method and intellectual curiosity has been my inspiration to pursue medical research. I would also like to express my appreciation to the Meakins-Christie fellows and staff for making the last three years a very enjoyable experience. In particular, I would like to thank Drs. James G. Martin, David H. Eidelman and Mara Ludwig for their wisdom and guidance. I also wish to thank my friends, Eve, Raj and David for all the good times and more importantly hope that our friendships will last forever. I would also like to take this opportunity to express my gratitude to my postdoctoral supervisor, Dr. Michelle Letarte, for all her help and understanding.

I would especially like to thank my parents for their unconditional love and support. I will always admire their courage and determination in their relentless pursuit to provide their best for my sister and I. I also wish to thank my sister, Nazli, for believing in me and always looking out for the best interests of her "little brother". Finally, I would to like to thank my fiancée Maria for all her love, affection and understanding and for always keeping me grounded.

ABSTRACT

The overall aim of this thesis was to analyze the expression of key vasoregulatory genes in the vascular endothelium and smooth muscle following hypoxia. Hypoxia caused a reversal in endothelial cell function such that the vascular endothelium enhanced α-agonist induced contractions. Downregulation of endothelial nitric oxide synthase (eNOS) and impaired endothelium-dependent vasodilation were observed in the aortas of rats exposed to hypoxia for 12 and 48 hours. Following stimulation of aortic rings with acetylcholine, cyclic GMP and nitrite levels were also reduced. Decreased endothelial NO release may impair the vascular responses which defend vital organ oxygenation. This decrease in the capacity for vasodilator synthesis following hypoxia cannot by itself, however, mediate the observed enhancement of contractility. An increase in aortic endothelin-1 (ET-1) mRNA and protein was localized to the vascular endothelium. Treatment with the ET-1 receptor antagonist, BQ123, mimicked endothelial denudation and confirmed that locally secreted ET-1 accounts for endothelium-dependent enhancement of contractility. Concurrently, hypoxia caused an endothelium-independent impairment of smooth muscle reactivity to α-adrenoreceptor activation. Despite a decrease in eNOS in the aorta of rats exposed to hypoxia, Ca^{2+}-dependent NOS activity remained unchanged suggesting the upregulation of neuronal NOS (nNOS). In endothelium-denuded aortic rings from hypoxic rats, L-NAME enhanced the contractile response to phenylephrine. In addition, it normalized the hypoxia-induced hypocontractility in intact aortic rings. The presence of nNOS was confirmed by Western blot analysis, immunohistochemistry, *in situ* hybridization and RT-PCR. Rat nNOS mRNA transcript bearing the alternately spliced exon 1b was shown to be expressed in the medial layer of aortas from hypoxic rats and in vascular smooth muscle cells cultured under hypoxic conditions. Cloning and analysis of the 5' regulatory sequence upstream of exon 1b revealed putative binding sites for hypoxia inducible factor-1 (HIF-1) and activating protein-1 (AP-1). These novel observations indicate that nNOS is a hypoxia-inducible gene expressed in vascular smooth muscle and plays an important role in hypoxia-induced vascular hypocontractility. The findings presented in this thesis

demonstrate that the reciprocal regulation of eNOS and ET-1 in the vascular endothelium and the upregulation of nNOS in smooth muscle play previously unidentified and significant roles in the vascular alterations associated with hypoxia.

RÉSUMÉ

L'objectif premier de cette thèse est d'étudier l'expression des gènes vasorégulateurs de l'endothélium vasculaire et du muscle lisse suite à l'hypoxie. L'hypoxie a causé une alteration au niveau de l'endothélium qui a eu comme effet d'amplifier les contractions induites par la stimulation des récepteurs α-adrénergiques. Nous avons observé une diminution dans l'expression du synthase de monoxide d' azote endothéliale (eNOS) et une atténuation de la vasodilation dépendante de l'endothélium dans l'aorte de rats soumis à l' hypoxie pour une période de 12 et 48 heures. Suite à une stimulation à l'acétylcholine, les niveaux de cGMP et de nitrite furent réduits. La diminution de la production de NO par l'endothélium pourrait atténuer les réponses vasculaires responsables de l'oxygénation des organes vitaux. Cette diminution de la capacité de synthèse de substances vasodilatatrices suite à l'hypoxie, ne peut expliquer à elle seule l'augmentation de contractilité observée. Une élévation de synthèse d'endothéline-1 (ET-1) a été localisée au niveau de l'endothélium. Un traitement avec l'antagonistes du récepteur de ET-1, BQ123, fut équivalent à la dénudation endothéliale de l'aorte, confirmant que ET-1 secrétée localement serait responsable de l'augmentation de contractilité. Simultanément, l'hypoxie a induit une réduction de la réactivité du muscle lisse à une activation α-adrénergique, independament de l'endothélium. Malgré une réduction de eNOS dans l'aorte de rats soumis à l'hypoxie, l'activité NOS Ca^{2+}-dépendante, demeure inchangée, suggérant une augmentation de NOS neuronal (nNOS). Dans l'aorte dépouillée d'endothélium chez les rats hypoxiques, L-NAME augmenta la réponse contractile à la phényléphrine. De plus, il normalisa l'hypocontractilité dans l'aorte intacte. L'expression de nNOS fut confirmée par buvardage électrophorétique, immunohistochimie, hybridization *in situ* et amplification par RT-PCR. L'expression d'un cDNA contenant l'exon alternatif 1b a été démontrée dans l'aorte des rats soumis à l'hypoxie. La séquence de la région régulatrice, qui précède cet exon, a révélé des sites potentiels de liaison des facteurs de transcription HIF-1 et AP-1. Nos résultats démontrent que nNOS est un gène induit dans le muscle lisse suite à l'hypoxie et qui par conséquent joue un rôle important dans l'hypocontraction vasculaire associée à cette condition. Les decouvertes presentées dans cette thèse demontrent que la régulation

réciproque de eNOS et ET-1 dans l'endothélium vasculaire et l' induction de nNOS dans le muscle lisse jouent donc des rôles importants dans les altérations vasculaires associées à l'hypoxie.

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Contributions of Authors

Chapter 2 is presented in the form of an original paper that has been recently published: Downregulation of Endothelial Nitric Oxide Synthase in Rat Aorta Following Hypoxia *In Vivo*. Mourad Toporsian, Karuthapillai Govindaraju, Mohammed Nagi, David Eidelman, Gaetan Thibault and Michael E. Ward. *Circ Res* 2000 86 (6): 671-675. I was the principal author of this paper and performed all of the experiments, except for in vitro contractility experiments that were performed by a summer student, Mr. Mohammed Nagi, and nitrate measurements made by Dr. Karuthapillai Govindaraju, a research associate supervised by Dr. David Eidelman. I performed the cGMP radioimmunoassays at l'Institut de Recherche Clinique de Montreal (IRCM) where I was co-supervised by Dr. Gaetan Thibault. Dr. Michael E. Ward supervised the entire work presented in this paper and is the corresponding author.

Chapter 3 is presented in the form of an original paper to be submitted: Increased Endothelin-1 Expression in Rat Aorta Mediates Endothelial Enhancement of α-Agonist-Induced Contractions Following Hypoxia *In Vivo*. Mourad Toporsian, Peter Cernacek and Michael E. Ward. I am the principal author and performed all of the experiments, except for radioimmunoassays for endothelin-1 and thromboxane B_2 which were done by Dr. Peter Cernacek. Dr. Michael E. Ward supervised the entire work presented in this paper and is the corresponding author.

Chapter 4 is presented in the form of an original paper to be submitted: Smooth Muscle Specific Induction of Neuronal Nitric Oxide Synthase Impairs Aortic Contractility Following Hypoxia *In Vivo*. I am the principal author and performed all of

the experiments. Dr. Michael E. Ward supervised the entire work presented in this paper and is the corresponding author.

Chapter 1

Introduction

Hypoxia elicits systemic vascular reflexes, mediated primarily by the sympathetic nervous system, which enhance oxygen extraction and redistribute the available oxygen supply to vital organs [1-3]. The maintenance of vital organ function, during reductions in systemic oxygen delivery, depends on the degree to which these responses are preserved while the vascular tissues themselves are rendered hypoxic. We have previously demonstrated that the exposure of rats to hypoxia for 12 and 48 hours has profound effects on the regulation of resistance arteriolar tone [37] and arterial [4] contractility. The abnormalities in vascular function reported in these studies include: 1) impaired endothelium-dependent vasoregulation; 2) loss of arteriolar myogenic responsiveness; and 3) impaired smooth muscle contraction in response to α-adrenoreceptor stimulation in both resistance and conductance vessels. These alterations in the function of both endothelial and smooth muscle cells develop within the first 12 hours of hypoxia, progress over the ensuing 48 hours and persist for at least 12 hours after the restoration of normoxia [4]. These abnormalities will limit autoregulation of tissue blood flow and impair circulatory responses to subsequent insults such as hypovolemia and hypotension due to hemorrhage or cardiac dysfunction. Furthermore, the failure to constrict upstream resistance arterioles during rises in venous or arterial pressure will lead to the inability to stabilize transcapillary fluid flux [5].

The overall aim of the studies presented in this thesis is to identify key vasoregulatory genes and their products that may account for the observed hypoxia-induced changes in aortic endothelium and smooth muscle functions following hypoxia *in vivo*. This will be accomplished by the use of standard molecular biological techniques coupled with pharmacological experiments in order to assess the functional

roles of specific vasoregulatory mediators in relation to vascular contractility following hypoxia.

In order to familiarize the reader with the topics covered throughout the thesis, an overview of the relevant literature will be presented. In the first part of the introduction, physiological concepts in normal tissue oxygenation as well as the circulatory adjustments to hypoxia and experimental models will be reviewed. Subsequently, general aspects of smooth muscle and endothelial functions will be covered with special emphasis on candidate vasoregulatory mediators implicated in the vascular dysregulation following hypoxia.

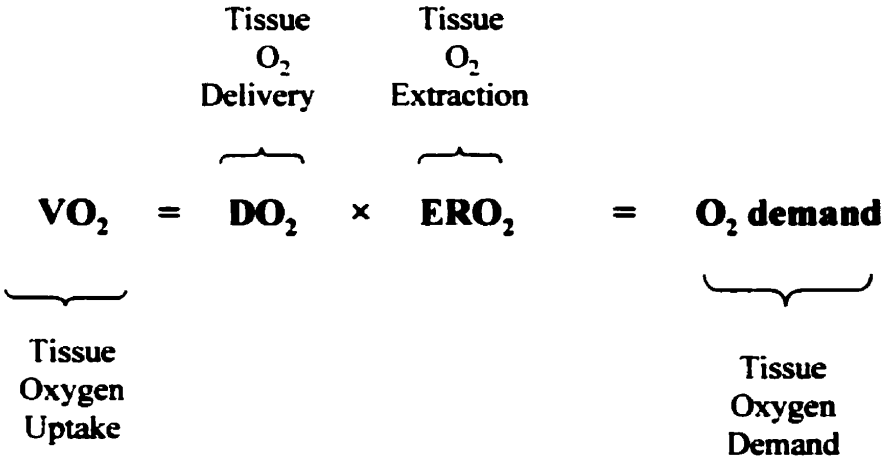
1.1 Tissue Oxygenation

Oxygen (O_2) plays a key metabolic role, yet except for oxymyoglobin found in skeletal muscle cells, this vital molecule is not stored in tissues. Oxidative metabolism must therefore depend on the constant delivery of O_2. In mammals and other vertebrates, O_2 is bound to the tetrameric protein hemoglobin (Hb) in red blood cells and transported from the lungs to the tissue capillaries via the cardiovascular system [6,7]. Since O_2 requirements vary among tissues, important vasoregulatory mechanisms have evolved to maintain the distribution of blood flow to organs in proportion to their metabolic demand [8,9]. According to Pfluger's law, oxygen uptake by tissues (VO_2) is normally dependent on metabolic demand and is not regulated or determined by oxygen availability [10-12]. In this circumstance, tissue oxygen uptake (VO_2) equals tissue oxygen demand and is determined by the product of tissue O_2 delivery (DO_2) and extraction (ERO_2) (Figure 1A)[13]. While DO_2 depends on the oxygen carrying capacity of blood and tissue blood

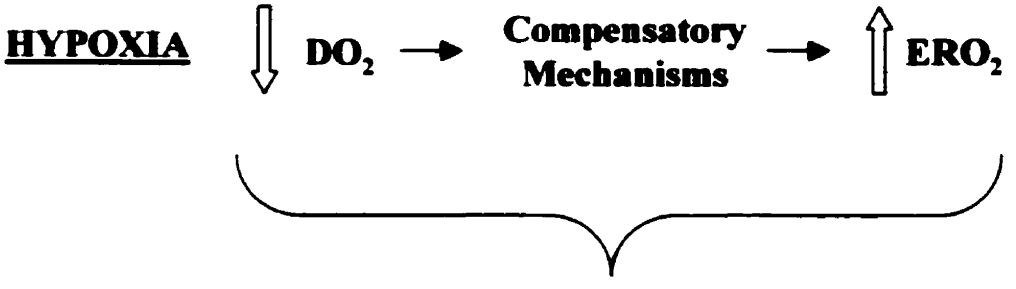
Figure 1.1

Relationship between O_2 consumption and metabolic demand.

A



B



VO_2 = O_2 Demand

flow, ERO_2 depends on the ability of tissues to utilize O_2. Hypoxia exists when either DO_2 or ERO_2 fall below the level normally required by cells to generate adequate amounts of adenosine triphosphate (ATP) and phosphocreatine necessary to maintain crucial biochemical processes. A reduction in DO_2 can be due to a decrease in blood hemoglobin saturation, reduced oxygen carrying capacity of blood or low tissue blood perfusion. Inadequate ERO_2, on the other hand, occurs during cyanide poisoning, because of the lack of an essential cofactor in cellular metabolism or during impaired microcirculatory regulation as occurs during sepsis.

In a setting of constant metabolic demand, a drop in DO_2, leads to a compensatory increase in ERO_2 in order to maintain a constant VO_2 (Fig. 1b). These increases in ERO_2 are however effective until a critical point (DO_{2crit}) below which VO_2 is rendered supply-dependent and declines with further decreases in DO_2. When DO_2 is less than DO_{2crit}, oxygen consumption is regulated by the availability of oxygen and not by tissue metabolic demand, thus causing an oxygen deficit [13,14]. A prolonged or sufficiently large oxygen deficit will eventually deplete intracellular stores of high-energy phosphates and thus cause derangements in key biochemical processes that may ultimately lead to cell death.

1.2 Circulatory Adjustments to Hypoxia

Hypoxia elicits adaptive circulatory adjustments, which redistribute blood flow to vital organs and optimize intraparenchymal flow distribution. These vascular responses increase ERO_2 in order to maintain VO_2 and are a consequence of the simultaneous activation of both central and local factors [14]. While central factors mediated by the

activation of the sympathoadrenal system increase vasoconstrictor tone, local factors produced by the cellular components of the arterial wall and surrounding tissue parenchyma tend to oppose this effect and cause dilation of metabolically active vascular beds.

1.2.1 Central Factors

A drop in arterial O_2 tension depolarizes the specialized chemoreceptor cells of the carotid and aortic bodies which then activate the vasomotor center of the brain to increase sympathetic vasoconstrictor tone [15]. The magnitude of this chemoreceptor reflex is not an all-or-none phenomenon and can be potentiated by several other factors present during hypoxia including acidosis [16], the surge in catecholamine release from the adrenal glands [15,17,18] and the activation of the baroreflex by hypotension [19]. This centrally mediated response to increase neurohumoral sympathetic tone during hypoxia tends to increase arterial pressure and redistribute blood flow to vital organs [1,2].

Normally, blood flow is distributed such that each organ receives a fraction of whole-body DO_2 in proportion to its metabolic demand. During hypoxia, those organs that are incapable of further increasing their ERO_2 such as the brain and the heart receive more blood flow and therefore a larger fraction of whole-body DO_2 while organs such as the kidneys and the splanchnic viscera receive a smaller fraction. In fact, it has been demonstrated that the redistribution of whole-body DO_2 is a determinant of DO_{2crit} and therefore will enhance the ability of the organism to maintain VO_2 in the supply-independent state [20,21]. Moreover, during hypoxia, neurohumoral adrenergic vasoconstriction has been shown to be necessary to improve the efficiency in extracting

O_2 and thereby promoting an increase in survival time [21]. It has been proposed that this increase in sympathetically mediated vasoconstrictor tone maintains arteriolar blood flow to metabolically active tissues and reduces blood flow to non-active ones [22]. This acts to prevent the vascular steal of blood flow away from tissues with high metabolic demand. The preservation of vascular smooth muscle reactivity to adrenergic stimuli while the vessels themselves are rendered hypoxic, therefore, is crucial to maintain adequate tissue oxygenation in proportion to metabolic demand and thereby promote survival.

1.2.2 Local Factors

Even though arteriolar blood flow is directed towards specific organs in proportion to their metabolic demand, there is no guarantee that the capillary bed actually receives this blood proportionally to tissue needs. Local factors including the release of important vasoactive substances modulate the density of perfused capillaries within tissues. This local control is constituted by three major components: 1) myogenic control involving smooth muscle cells; 2) a flow-dependent response which involves the endothelium-derived relaxing factors; and 3) metabolic regulation involving parenchymal and vascular cells through the release of metabolites (e.g. adenosine).

The local regulation of vascular tone refers to the intrinsic contraction of vascular smooth muscle cells and its modulation by endothelium-derived vasoactive substances and cellular metabolites [23]. The myogenic response, first described by Bayliss in 1902 [24], is the ability of smooth muscle to contract and relax in response to increases and decreases in transmural pressure, respectively. Blood vessels are continuously exposed to changes in transmural pressure and, therefore, the myogenic response always

contributes to the local control of vascular tone. The actual strength of the response, however, depends on the vessel diameter. Large (aorta) and very small vessels display a very weak myogenic response, while vessels of intermediate diameter have the largest response [25]. The vascular endothelium secretes both vasodilators and vasoconstrictors and thereby modulates vascular tone by varying the pressure sensitivity or, in other words, the myogenic responsiveness of blood vessels. Cellular metabolites can modulate myogenic responsiveness through a direct effect on smooth muscle or through the release of endothelium-derived dilators. Conversely, the myogenic response also contributes to the vasoconstriction and resulting increase in transmural pressure by endothelium-derived constrictors indicating that these control mechanisms are interdependent [23].

In metabolically active tissues, a drop in interstitial PO_2 leads to vasodilation which competes with sympathetic vasoconstrictor tone to increase tissue blood perfusion. This mechanism also results in localized alterations in vascular tone throughout the cardiovascular system to enable the redistribution of blood flow in proportion to O_2 demand. While the loss in vascular smooth muscle tone in vessels from metabolically active tissues may be an adaptive response to increase tissue blood perfusion during short hypoxic episodes, the persistence of this abnormality would have maladaptive effects. Hyporeactive blood vessels would lose the capacity to autoregulate tissue blood flow and also fail to stabilize transcapillary fluid flux during increases in venous or arterial pressure since changes in exchange vessel pressure are normally buffered by the ability of upstream arterioles to adequately constrict in response to rises in intraluminal pressure. With the persistence of hypoxia, certain alterations in vascular function would therefore not only limit the ability of the systemic circulation to optimally distribute the available

oxygen supply, but also handicap vascular responses to superimposed stresses such as sepsis, cardiac dysfunction, a sudden increase in tissue metabolic rate or hypovolemia due to hemorrhage. During such instances, failure to properly readjust the distribution of blood flow would increase the severity of the hypoxic episode despite any further deterioration in DO_2 and thereby significantly contribute to organ system dysfunction.

This thesis will focus on the molecular alterations in endothelial and smooth muscle functions that occur following exposure of rats to hypoxia *in vivo*.

1.3 Experimental Models of Hypoxia

As with other physiological stimuli, the characteristics of a hypoxic response vary with the duration of exposure. Most studies of the cardiovascular effects of hypoxia have involved either acute or chronic exposures. An acute exposure generally occurs over a brief time frame of seconds to minute whereas a chronic exposure extends over a period of months. Cellular responses to an acute and chronic hypoxic episode range from rapid changes in carbohydrate metabolism to permanent remodeling of vascular tissues, respectively. At the molecular level, acute hypoxia is characterized by posttranslational modification of pre-existing proteins and other macromolecules via reactions that involve, for example, phosphorylation/dephosphorylation or oxidation/reduction [26]. These modifications rapidly alter the activity of target molecules such as ion channels, enzymes as well as structural proteins and thereby allow cells to quickly adapt to the hypoxic microenvironment. For instance, hypoxia is known to inhibit K^+ channel activity that results in membrane depolarization and the influx of Ca^{2+} through voltage-dependent

calcium channels [27]. These sorts of changes are quickly induced during acute hypoxia, and are immediately reversed upon restoration of normoxia. On the other hand, a chronic hypoxic episode involves complex changes in gene expression and therefore alterations in cellular phenotype and function are likely to persist even after the removal of the hypoxic stimulus.

1.3.1 Acute Hypoxia

During acute hypoxia *in vivo*, vasodilatation is often observed in most vascular beds including the cerebral, coronary, skeletal and gastrointestinal circulations [28,29]. The response of arteries to acute hypoxia *in vitro*, however, can be endothelium-dependent dilation [30,31], endothelium-dependent constriction [32] and varying degrees of impaired smooth muscle contractility [33]. The disparity between *in vitro* and *in vivo* results indicates that blood vessels have distinct responses to acute hypoxia that are masked by the action of metabolic mediators released by the surrounding parenchyma *in vivo*. These metabolites are generally vasodilator substances including adenosine (ADP), carbon dioxide, lactic acid, histamine potassium ions, and hydrogen ions. ADP is by far the most important of the local vasodilatory metabolites for controlling local blood flow [6]. Interestingly, despite the variability in the effects of acute hypoxia on vascular tone, the restoration of normoxia always immediately reverses the hypoxic response.

1.3.2 Chronic Hypoxia

Studies on the effects of chronic hypoxia on systemic vasoreactivity *in vivo* have also given rise to conflicting results. Vascular responses to contractile agonists have been

shown to be increased [34] in some studies and impaired [35] or unchanged [36] in others. This variability may be due to differences in animal species, method of anesthesia and route of drug administration (intravenous versus intra-arterial). Furthermore, the effects of chronic hypoxia on vascular responses become increasingly difficult to interpret following months of exposure. Organisms exposed to hypoxia for extended periods become progressively acclimatized to the low oxygen tension through various means: 1) increased pulmonary ventilation; 2) alteration in the hemoglobin dissociation curve by 2,3-diphosphoglycerate; 3) increased red blood cells; 4) increased diffusion capacity of the lungs; 5) increased capillarity of tissues; and 6) increased ability of cells to use oxygen despite its low partial pressure [6]. Given these adaptive mechanisms to diminish the severity of the hypoxic exposure, it is difficult to dissociate the direct effects of hypoxia on systemic vascular responses from the secondary effects as a result of acclimatization to low oxygen tensions.

1.3.3 Hypoxia of Intermediate Duration

A clinically relevant duration of exposure to hypoxia is one that occurs over a time course of hours to days since the cardio-respiratory illnesses that result in admission to the adult intensive units, typically evolve over this time frame. Furthermore, this time interval represents a very vulnerable period for the organism since alterations in gene expression have had sufficient time to manifest themselves and affect cellular function, while it is much too early for more complex and timely mechanisms of acclimatization to have fully taken effect. The organism is, therefore, still dependent on its ability to preserve the reactivity of its vessels to adrenergic stimuli while the expression of certain

proteins, which may negatively affect vasoreactivity to such stimuli, may have had sufficient time to manifest themselves. This time interval thus provides an ideal opportunity to identify and study the mediators involved in the vascular alterations that occur during hypoxia.

We have previously demonstrated that the exposure of rats to 12 and 48 hours of hypoxia has profound effects on the regulation of resistance arteriolar tone [37] and arterial [4] contractility. The abnormalities in vascular function reported in these studies include: 1) impaired endothelium-dependent vasoregulation; 2) loss of arteriolar myogenic responsiveness; and 3) impaired smooth muscle contraction in response to adrenoreceptor stimulation in both resistance and conductance vessels. These alterations in both endothelial and smooth muscle cell function develop within the first 12 hours of hypoxia, progress over the ensuing 48 hours and persist for at least 12 hours after the restoration of normoxia. Interestingly, since similar responses were observed in both large vessels and resistance arterioles, the aorta was used in all of the studies presented in this thesis because this vessel provides sufficient mRNA and protein for all of the biochemical assays and, unlike arterioles, it can be easily manipulated for organ bath contractility experiments.

Endothelial Abnormality. The vascular endothelium normally exerts a negative influence on vascular tone and attenuates α-agonist-induced contractions. Interestingly, these studies indicated that hypoxia causes a reversal in vascular endothelial function such that the endothelium becomes a net vasoconstrictor agency and enhances α-agonist-induced contractions. This is contrary to what is observed during acute hypoxia where the loss of vascular tone is endothelium-dependent and is quickly reversible by normoxia

[30,31]. The results of these studies suggest that hypoxia may induce the loss of a potent endothelium-derived dilator and concomitantly induce the expression of a potent endothelium-derived constrictor. It is likely that this change in endothelial function compensates for the loss in smooth muscle contractility at the expense, however, of losing important endothelium-dependent vasoregulatory mechanisms.

Smooth Muscle Abnormality. With respect to the smooth muscle, hypoxia induced a loss in smooth muscle contractility and this effect was not dependent on the endothelium. This suggests that hypoxia most likely causes alterations in vasoregulatory genes whose products are contractile proteins and/or proteins that interfere with normal contraction. At least initially, a reduction in smooth muscle contractility may be part of an adaptive response to increase blood flow and maintain oxygen transport to vital organs. With the persistence of hypoxia, however, the inability to actively regulate the systemic circulation during superimposed stresses will eventually have deleterious effects.

The hypoxia-induced abnormalities at the level of the vascular endothelium and smooth muscle will limit autoregulation of tissue blood flow and impair circulatory responses to subsequent insults such as hypovolemia and hypotension due to hemorrhage or cardiac dysfunction. Furthermore, the failure to constrict upstream resistance arterioles during rises in venous or arterial pressure will lead to the inability to stabilize transcapillary fluid flux [5]. An understanding of the molecular mediators involved in the vascular responses to hypoxia may enable the design of specific therapeutics geared towards the amplification of the adaptive responses to hypoxia and the inhibition of those that are pathogenic. The overall aim of this thesis is the identification of these vasoregulatory genes and their products that may account for the observed hypoxia-

induced changes in aortic endothelial and smooth muscle cell function following hypoxia *in vivo*.

General aspects of smooth muscle and endothelial functions will now be discussed with special emphasis on candidate vasoregulatory mediators that may play a role in the vascular dysregulation following hypoxia.

1.4 Smooth Muscle

Despite little doubt that reductions in oxygen tension affect smooth muscle contractility, its mechanisms have been the focus of numerous studies and yet are still not known with certainty. Given that hypoxia causes vasoconstriction in pulmonary blood vessels and vasodilation in most systemic vessels, the mechanisms are likely to be diverse. Several theories to account for the loss of smooth muscle contractility in response to hypoxia have been proposed and can be generally divided into those involving energy limitation and those where the mechanisms regulating excitation-contraction coupling are impaired. In the first category, hypoxic relaxation of smooth muscle is believed to be due to the limitation on cellular ATP synthesis, which compromises actin-myosin ATPase activity and leads to impaired contractility. Vascular smooth muscle ATP content is, however, well buffered and even severe deprivations in oxygen cause little or no change in the intracellular ATP concentration.[38,39]. Considering our previous observations that the impairment in smooth muscle reactivity was not immediately reversed upon restoration of normoxia, we suggest that mechanisms that interfere with excitation contraction coupling in smooth muscle are likely affected.

The mechanisms regulating excitation-contraction coupling in vascular smooth muscle contraction are very complex and, for the purpose of this thesis, will only be described briefly.

1.4.1 Smooth Muscle Contraction

The principal mechanisms that initiate contraction and relaxation in smooth muscle are the rise and fall of intracellular Ca^{2+} levels, respectively [40,41]. Calcium binds calmodulin (CaM) and this complex activates myosin light chain kinase (MLCK), which in turn, phosphorylates the 20kD regulatory light chain of myosin (LC_{20}). The phosphorylation of LC_{20} at serine 19 [42] results in a conformational change that exposes the active site of the myosin head and thus permits the binding of actin. This allows ATP hydrolysis by myofibrillary ATPase and initiates cross-bridge cycling. Unlike striated muscle myosin which is always in an activated state, smooth muscle myosin remains inactive until it is phosphorylated by the Ca^{2+}-dependent MLCK [43].

LC_{20} phosphorylation is the key event in the regulation of smooth muscle contraction and is tightly controlled by the opposing activities of MLCK and myosin light chain phosphatase (MLCP). A decrease in intracellular calcium causes the inactivation of MLCK and is accompanied by dephosphorylation of LC_{20} by MLCP. Furthermore, intracellular mediators that modulate MLCP activity alter the sensitivity of the contractile apparatus to calcium [40]. Contractile agonists that act through molecules such as protein kinase C (PKC) [44], arachidonic acid [45] and rho kinase [46] inhibit the activity of MLCP and thereby enhance the sensitivity to the contractile stimuli. Conversely, certain vasodilators such as nitric oxide (NO) exert their effect by desensitizing the contractile apparatus to

Ca^{2+} through the activation of MLCP [47]. It is apparent that NO-mediated decrease in vascular smooth muscle $[Ca^{2+}_i]$ and an increase in MLCP activity would lead to a decrease in myosin phosphorylation and ultimately result in vasorelaxation.

1.4.2 Excitation-Contraction Coupling

Stimuli activate smooth muscle by at least four mechanisms: 1) Contractile agonists or increases in extracellular $[K^+]$ ($[K^+]_o$) cause depolarization and increase Ca^{2+} influx through the activation of L-type Ca^{2+} channels; 2) Agonists induce the release of Ca^{2+} from intracellular stores; 3) Agonists stimulate both voltage-dependent and -independent Ca^{2+} channels, which together lead to an increase in Ca^{2+} entry more than that expected from the degree of depolarization; and 4) Agonists increase contractile force at a given intracellular concentration which is also known as Ca^{2+} sensitization [43]. While the first mechanism is electromechanical coupling since the contraction involves a change in membrane potential, the other three are forms of pharmacomechanical coupling since the contraction is larger than that that would be expected from the change in membrane potential. The first three mechanisms elicit contraction by changing $[Ca^{2+}_i]$, while the last one does not alter $[Ca^{2+}_i]$ (Ca^{2+} sensitization). Normally, agonists contract smooth muscle by some or all of these mechanisms while vasorelaxants interfere with these mechanisms. Phenylephrine, the contractile agonist used in all of the studies presented in this thesis, activate smooth muscle contraction through IP_3 (inositol triphosphate)-mediated Ca^{2+} release [48], an enhancement of myofilament Ca^{2+} sensitivity [49], and to some extent, an increase in Ca^{2+} influx due to the activation of L-type Ca^{2+} channels [50]. It follows that hypoxia-induced alterations in these pathways or the

induction of a mediator that inhibits these pathways would lead to a loss in smooth muscle reactivity to phenylephrine.

The properties of the vascular endothelium with special emphasis on the major vasodilators and vasoconstrictors that it secretes will now be discussed.

1.5 Vascular Endothelium

The vascular endothelium consists of a monolayer of endothelial cells that form the luminal surface of blood vessels and are in direct contact with the underlying medial layer of vascular smooth muscle. This optimal positioning of the endothelial cell at the interface between the circulating blood and the smooth muscle makes it an ideal sensor and signal transducer of the vascular microenvironment. In 1980, the demonstration by Furchgott and Zawadski that the endothelium contributes to the control of vascular tone gave rise to a new concept in the regulation of vascular function [51]. In response to chemical and mechanical stimuli, the vascular endothelium secretes an array of substances that play an important role in the regulation of vascular tone, blood fluidity and vascular architecture [52]. These endothelium-derived mediators maintain a delicate balance between vasoconstriction and vasodilation, coagulation and blood fluidity, and the promotion and inhibition of vascular growth. Overall, the normal vascular endothelium favors vasodilation and inhibits thrombosis and cellular proliferation. Damage to or removal of this monolayer of endothelial cells is known to increase basal vascular tone and promote platelet aggregation and smooth muscle cell proliferation [53].

Unlike neurons and cardiomyocytes whose viability is threatened with severe hypoxia, endothelial cells can maintain their integrity at low oxygen tensions (8-12 torr) over an extended time frame. They mainly accomplish this by relying on glycolytic ATP synthesis and having a large capacity to increase this metabolism. Even after exposure to 48 hours of hypoxia, endothelial cells still contain 70% of their normal ATP level and protein synthesis is maintained at 70-80% of the normoxic control [54]. This indicates that the changes observed following prolonged hypoxia are not likely due to the deterioration of cellular function as a result of cellular death, but rather to hypoxia-induced alterations of specific endothelium-dependent mechanisms.

We have previously demonstrated that the exposure to hypoxia for 12 to 48 hours causes a reversal in endothelial function that involves a loss of its normal inhibitory influence on vascular tone and the activation of mechanisms that enhance agonist-induced contraction. This may be an adaptive mechanism to counteract the observed simultaneous reduction in smooth muscle contractility following the hypoxic episode. It may be accounted for by an increased release in endothelium-derived vasoconstrictors and a decreased release in endothelium-derived vasodilators, which would unmask the basal constriction. The following is an overview of the main vasodilators and vasoconstrictors released by the vascular endothelium and their involvement in the regulation of vascular tone with special emphasis on the mediators that will be examined in the studies presented in this thesis.

1.6 Endothelium-Derived Vasodilators

In the systemic circulation, the principal endothelium-dependent vasodilators are nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factor (EDHF). Although it is well documented that both NO and PGI_2 play an important role in the regulation of vascular tone, it has been shown that these two factors alone cannot account for all of the observed endothelium-dependent vasodilatory responses. The existence of an endothelium-derived hyperpolarizing factor or EDHF has thus been proposed [55]. Although alterations in both PGI_2 and EDHF function/expression may be involved in the vascular dysregulation following hypoxia, these factors will only be described briefly and this section will focus instead on the potent endothelium-derived vasodilator, NO.

1.6.1 EDHF

While the identity of EDHF remains a mystery, recent evidence indicates that EDHF is a diffusible factor that causes vasorelaxation by hyperpolarizing underlying vascular smooth muscle. In many arteries, studies clearly suggest that EDHF is not NO or a prostanoid since inhibitors of NO synthases and cyclooxygenases do not attenuate endothelium-dependent hyperpolarization/relaxation of vascular smooth muscle, which is most likely mediated by the activation of potassium channels [56,57]. It has been suggested that EDHF is a product of cytochrome *P*-450 monooxygenase metabolism of arachidonic acid [56,58] and that the products of arachidonate that mediate this effect seem to be epoxyeicosatrienoic acids [56]. The role of these *P*-450 monooxygenase metabolites as possible EDHFs is partially supported by the use of cytochrome *P*-450 inhibitors.

Recently, it has been suggested that the release of cytochrome *P*-450 products by the endothelium contributes to the vasodilation of cerebral microvessels during hypoxia in newborn piglets [59]. These results, however, must be interpreted with caution since although these inhibitors abolish EDHF release from the endothelium [55], high concentrations of these compounds may also have non-specific effects. Finally, not all arteries produce an EDHF or NO/PGI_2-independent relaxation in response to agonist-induced stimulation. In general, the importance of EDHF as an endothelium-dependent relaxant decreases while that of NO increases as vessels become larger, ranging from the mesenteric circulation to the aorta. It has recently been suggested that the relative contribution of NO to endothelium-dependent relaxation was very prominent in the aorta, whereas that of EDHF was more important in the distal mesenteric arteries [60].

1.6.2 Prostacyclin

Prostacyclin is a dienoic bicyclic eicosanoid derived from membrane bound arachidonic acid metabolism. A heme-containing enzyme known as cyclooxygenase-1 catalyzes its biosynthesis in endothelial cells [61]. During acute hypoxia, the expression of endothelium-derived prostaglandins, in particular, prostacyclin (PGI_2), is upregulated and plays a role in hypoxic vasodilation [31]. With the persistence of hypoxia, this increase is no longer observed and there is in fact a decrease in the basal release of PGI_2 [62]. The loss of this important vasodilator during prolonged hypoxia may play a significant role in the observed reversal in endothelial cell function.

1.6.3 Nitric Oxide

Perhaps the most important endothelium-derived substance in relation to the regulation of vascular tone is nitric oxide (NO). In the normal vasculature, NO plays a very important role in endothelium dependent dilation through its potent relaxant effect on smooth muscle cells. This effect is predominantly mediated by the generation of cGMP in vascular smooth muscle although there are also cGMP-independent mechanisms of vasodilation [63]. Endothelium-derived NO has been shown to modulate myogenic autoregulatory responses [64], flow mediated dilation [65], active and reactive hyperemia [66,67] as well as hypoxic vasodilation in certain vascular beds [68]. Due to its involvement in these mechanisms, a central role has emerged for endothelium-derived NO in maintaining oxygen supply in proportion to metabolic demand. This, in turn, makes the characterization of its production and release from the vascular endothelium during reductions in oxygen supply very appealing.

The pioneering study by Furchgott in 1980 indicated that the relaxation of vascular smooth muscle in response to acetylcholine was dependent on the anatomical integrity of the endothelium and suggested the existence of a labile endothelium-derived relaxing factor (EDRF) [51]. Subsequently, several lines of evidence lead to the concept that this EDRF is in fact NO [69-71]. We now know that NO is a unique biological messenger molecule with a wide range of physiological functions including blood pressure control, regulation of platelet aggregation, cellular proliferation, neurotransmission and immune modulation [72]. Unlike most signaling molecules which require specific receptors, transporters or ion channels for entry into cells, NO is believed to be capable of freely diffusing from its source of synthesis across biological membranes

[73]. Because of its small size, rapid diffusibility and relatively low reactivity compared to other free radicals, it is presumed that the key to its regulation is at the level of synthesis. NO has a very short half-life and is known to interact avidly with heme proteins, sulfhydryl-containing proteins and oxygen-derived free radicals [73,74]. Its ability to nitrosylate tyrosine residues of various proteins and thus instantly modulate their activity makes NO attractive for study because it can modify the function of the cell where it is produced, as well as that of neighboring cells. However, its short half-life (4-8 seconds) complicates its detection. Most studies, therefore, have used indirect measurements of its more stable metabolites such as nitrite, nitrate, tyrosine nitration or the by-product L-citrulline as an index of NO production [52,53,74].

Mechanisms of NO-Mediated Vasorelaxation. The vasorelaxant effects of NO are predominantly mediated by 3'5'cyclic guanosine monophosphate (cGMP)-dependent mechanisms, although cGMP-independent mechanisms have also been reported [63].

The binding of NO to the heme moiety of soluble guanylate cyclase (sGC) in vascular smooth muscle dislocates the heme iron and thereby induces a conformational change in this enzyme, which gives rise to an increase in intracellular cGMP. cGMP, in turn, may either directly, or through the activation of PKG, mediate the relaxant effects of NO by lowering the smooth muscle intracellular Ca^{2+} concentration ($[Ca^{2+}_i]$), and by reducing the sensitivity of the contractile apparatus to $[Ca^{2+}_i]$ via the activation of MLCP[40].

There are numerous proposed mechanisms for NO and cGMP-mediated reduction in smooth muscle $[Ca^{2+}_i]$: 1) NO and cGMP -mediated hyperpolarization via the activation of large conductance Ca^{2+}-activated K^+ (BK_{Ca}) and K^+_{ATP} channels [75-77]; 2)

both cGMP and NO-mediated inactivation of L-type Ca^{2+} channel conductance[78]; 3) PKG-mediated activation of Ca^{2+}-ATPase pump on sarcoplasmic reticulum and plasma membrane to increase Ca^{2+} sequestration [79] and 4) PKG-mediated inhibition of IP_3 induced Ca^{2+} mobilization [80].

1.7 Nitric Oxide Synthases

1.7.1 Nomenclature

NO is synthesized by a family of cytochrome P-450-like enzymes known as nitric oxide synthases (NOS). These enzymes are flavohemeproteins that catalyze the formation of NO from the basic amino acid L-arginine [81-83]. Three NOS isoforms have been identified and each of these is the product of a distinct gene [84-86]. Their widely accepted nomenclature is based on the tissue or cell in which they were initially identified, namely endothelial cells (eNOS), macrophages (macNOS) and neuronal tissues (nNOS). Macrophage NOS was renamed the inducible NOS (iNOS) when it was realized that the expression of this isoform is not only specific to macrophages, but can also be "induced" in a wide variety of tissues including smooth muscle in response to pro-inflammatory cytokines. Until recently, the eNOS and nNOS were considered to be constitutive enzymes while the expression of iNOS was induced by cytokines. Yet, as novel information is provided, this nomenclature is also somewhat misguiding. Current evidence demonstrates that the expression of both eNOS and nNOS can in fact be inducible. As predicted by the identification of six shear stress response elements in the promoter of eNOS, the expression of this isoform is upregulated by endothelial shear stress due to flow [87]. Furthermore, treatment with estradiol and pregnancy have also been

shown to upregulate both eNOS [88,89] and nNOS expression [90]. Transection of the sciatic nerve [91] and treatment with neurotoxins also increase the expression of nNOS in the ipsilateral dorsal root ganglion [92] and hippocampus [93], respectively. Finally, neural ischemia induces the expression of nNOS in neurons indicating that this isoform may indeed be hypoxia-inducible. Conversely, the inducible NOS may also function as a constitutive enzyme under certain physiological conditions. The iNOS has been detected in human bronchial epithelium [94] and alveolar macrophages [95] in the absence of inflammation. The designation of a NOS isoform as either constitutive or inducible is therefore misleading.

1.7.2 Enzymatic Activity

All three NOS isoforms exist as homodimers and share a similar overall catalytic scheme that involves the five-electron oxidation of the terminal guanidino nitrogen of L-arginine to form NO and L-citrulline. This complex reaction also requires molecular O_2 and NADPH as co-substrates, Ca^{2+} bound calmodulin (CaM), as well as redox cofactors such as enzyme-bound heme, FAD, FMN and tetrahydrobiopterin (BH_4) [83]. NO synthesis by all three NOSs depends on the capacity of these enzymes to bind the calcium regulatory protein CaM. Both eNOS and nNOS are Ca^{2+} dependent since they require increases in resting intracellular Ca^{2+} concentrations ($[Ca^{2+}_i]$) in order to bind CaM and become fully activated. In contrast, iNOS appears to bind CaM with extremely high affinity even at the low $[Ca^{2+}_i]$ available in resting cells and is thereby characterized as Ca^{2+} independent. The activities of both eNOS and nNOS are temporally regulated by intracellular calcium transients via the triggering effect of CaM [72]. This triggering role of CaM has been explained by a switch mechanism between the reductase and the

oxygenase domains. The NOS flavins accept electrons from NADPH (reductase domain) and, upon CaM binding, transfer them to the heme iron (oxygenase domain) which enables the heme iron atom to bind oxygen and catalyze L-arginine oxidation [83].

Molecular oxygen is an essential co-substrate for all NOS isoforms. The affinity of molecular oxygen for these enzymes is different with respect to each isoform and therefore the extent to which their activities may be compromised during conditions of low oxygen tension is likely to vary accordingly. The iNOS has the lowest K_m (6.3 μM, 12.93 mmHg) for oxygen followed by eNOS (K_mO_2=7.7 μM, 15.08 mmHg) and nNOS (K_mO_2=23.2 μM or 45.45 mmHg). This indicates that the activity of the nNOS, would be most compromised during physiologically relevant conditions of low oxygen tension. In fact in our model of hypoxia, the arterial PO_2 averaged 38 mm Hg (range 35-42) and therefore under these circumstances nNOS would at best be operating at or below $_{1/2}V_{max}$. Given the appropriate conditions, however, even low levels of NO can have pathological effects. Numerous studies have demonstrated an increase in superoxide (O_2^-) generation during hypoxia [96-100]. NO can readily react with O_2^- and form the very powerful oxidant, peroxynitrite *in vivo* [101-107]. Peroxynitrite can attack many types of biological molecules, thus altering overall cellular function and possibly contributing to the observed vascular dysregulation during hypoxia.

1.7.3 Endothelial Nitric Oxide Synthase

The endothelial nitric oxide synthase (eNOS) is a 140kD calcium/calmodulin dependent NOS isoform predominantly expressed in endothelial cells and in the normal vasculature, where it is the only source of NO. Endothelial release of nitric oxide (NO), has been shown to modulate myogenic autoregulatory responses [64] and to mediate flow-

dilation of resistance arterioles [65], active hyperemia [66], reactive hyperemia [67] and, in some vascular beds, hypoxic vasodilation [68]. Thus, a central role is emerging for endothelial production of NO in maintaining the balance between tissue oxygen supply and metabolic demand. Interestingly, the expression of eNOS appears to be under the regulatory control of oxygen tension. This finding is significant because it provides a mechanism by which the capacity of vascular beds to autoregulate their blood flow may be altered during episodes of reduced oxygen availability. However, these molecular studies have been conducted in cultured endothelial cells and have yielded conflicting results. Cultured endothelial cells from umbilical [108] and saphenous[109] veins, and from bovine pulmonary artery [110] and aorta [111] exposed to hypoxia (0% O_2) for 24 hours have shown a downregulation of eNOS protein and mRNA. In contrast, hypoxic incubation (1% O_2 for 24 hrs) of bovine aortic endothelial cells has resulted in an increase in eNOS protein [112] expression. Upregulation of eNOS protein and activity has also been demonstrated in early passage porcine coronary arteriolar endothelial cells after hypoxic exposures of 30 minutes to 4 hours in duration [113]. This variability in results may be related to differences in the origin of endothelial cells as well as the duration and severity of the hypoxic episode. Furthermore, the method of extraction of these cells as well as their maintenance in a differentiated state in an artificial environment may have profound effects on their responsiveness to hypoxia. As a result of the lack of consistent data in previous studies, we are still left with the question of whether or not *in vivo* hypoxia alters eNOS expression and, if so, its consequences on the regulation of vascular tone.

1.7.4 Inducible Nitric Oxide Synthase

The inducible NOS (iNOS) is a 130kD cytosolic protein which is able to synthesize high levels of NO. A wide variety of cells, including vascular smooth muscle cells are capable of expressing the inducible NOS (iNOS) in response to pro-inflammatory cytokines [114,115] and the expression of this Ca^{2+}-independent isoform is known to contribute to impaired vascular reactivity in certain disease states [116,117]. Recently, a binding site for the hypoxia-inducible factor-1 (HIF-1) [118-122], a nuclear protein that mediates the expression of numerous hypoxia-inducible genes, has been identified in the promoter region of the iNOS gene [123]. Although hypoxia alone does not elicit functional iNOS expression, it strongly potentiates iNOS induction by IL-1 [124,125] and IFN-γ [126]. In fact, the release of these pro-inflammatory cytokines is markedly increased during systemic hypoxia [127-133], and therefore suggests the possibility that iNOS may be expressed in vascular smooth muscle following hypoxia *in vivo*.

1.7.5 Neuronal Nitric oxide Synthase

The largest and most widely distributed NOS isoform is the Ca^{2+}-dependent neuronal NOS (nNOS). DNA analysis of human nNOS has revealed a complex genomic locus consisting of 29 exons distributed over at least 200 kb [134]. The regulation of nNOS transcription and wide tissue distribution is characterized by the use of multiple promoters [135-137]. This results in mRNA species with different 5'untranslated first exons alternatively spliced into a common exon 2 containing the translation initiation codon (ATG) and leaving the nNOS protein structure unaltered. There are at least three different first exons (1a, 1b and 1c) reported in the rat [138] and nine others in the human nNOS gene [136], suggesting the likelihood of nNOS expression in a variety of tissues

given the appropriate microenvironment. Previous studies have shown an increase in nNOS mRNA in central and peripheral neurons [139-142] as well as skeletal muscle [143] following hypoxia. While the expression of nNOS has not been reported in the smooth muscle of normal blood vessels, its expression has been demonstrated in human smooth muscle cells (SMCs) grown in culture [144], in atherosclerotic plaques [135] and in common carotid arterial smooth muscle of spontaneously hypertensive rats (SHR) [145]. Given that nNOS is a hypoxia-inducible gene in some tissues and that smooth muscle cells are capable of expressing this enzyme under certain conditions, it is possible that nNOS may also be expressed in vascular smooth muscle following hypoxia *in vivo* and play a role in the observed loss of vascular reactivity.

1.8 Hypoxia-inducible Transcription Factors

In general, the transcriptional activator hypoxia inducible factor-1 (HIF-1) is considered a master regulatory protein that induces the expression of numerous oxygen-regulated genes [119,122]. It is a heterodimeric transcription factor consisting of two basic-helix-loop-helix (bHLH) proteins of the PAS (per arnt sim) family termed HIF-1α and HIF-1β [120]. The HIF-1β subunit is also known as the aryl hydrocarbon nuclear translocator (ARNT) and can dimerize with other bHLH transcription factors of the PAS family. HIF-1α, on the other hand, is unique to HIF-1 and its expression is increased during reductions in cellular oxygen tension [121]. HIF-1 binds the consensus sequence 5'TACGTGCT3' in the erythropoietin enhancer, and this site-specific DNA binding activity facilitated its purification and the cloning of target genes [121]. The number of genes that are regulated by this transcription factor is continuously expanding and

includes genes whose products are involved in angiogenesis, energy metabolism, erythropoiesis, cellular proliferation and apoptosis, vascular remodeling and vasomotor responses [146]. Another transcription factor that is commonly associated with the induction of most hypoxia-inducible genes is activator protein-1 (AP-1). Hypoxia stimulates c-fos expression both *in vivo* [147]and *in vitro*[148]. The fos protein forms a heterodimer with another factor Jun or homodimers with fos and bind to the consensus AP-1 activator site 5' TGACTCA 3' [149]. Hypoxia has been shown to increase AP-1 activity and antisense oligomers to c-fos prevent this activation [150,151]. The presence of binding sites for these transcription factors in promoters of certain vasoregulatory genes have already been identified. They are present in the proximal promoter of iNOS and ET-1 [123,150,152].

1.9 Endothelium-derived Vasoconstrictors

The endothelium-derived constricting factor that has received the most investigation is the 21-amino-acid polypeptide, endothelin. The endothelin-1 (ET-1) isoform is principally released from vascular endothelial cells via the constitutive pathway in response to chemical [153,154] and mechanical [155] stimuli. The expression of ET-1 mRNA is increased in response to growth factors and cytokines such as thrombin [156] transforming growth factor β [157], tumor necrosis factor α [158], or with vasoactive substances [159,160] such as norepinephrine, angiotensin II, vasopressin and bradykinin.

ET-1 and its mRNA are also increased in endothelial cells cultured under hypoxic conditions [161]. Moreover, plasma ET-1 levels are increased in rats and humans during hypoxia and reductions in ambient oxygen tension increase ET-1 production in arterial

segments *in vitro* [162-166]. Increased endothelium-derived ET-1 in the systemic vasculature is, therefore, an attractive candidate for the mechanism mediating endothelium-dependent enhancement of agonist-induced contraction after exposure to hypoxia of intermediate duration.

In previous studies, however, an increase in preproET-1 mRNA could not be detected by Northern analysis in the systemic vasculature of rats following exposure to hypoxia despite an increase in plasma ET-1 concentrations [163,166]. It was concluded that production of ET-1 is not part of the systemic vascular response to physiologically relevant levels of hypoxia *in vivo*. Northern blotting is an insensitive method, however, by which to exclude the expression of a potent vasoconstrictor that may exert important effects at low concentrations. Furthermore, ET-1 production may be regulated at the level of translation or during post-translational enzymatic processing [167,168] as well as at the level of transcription. Consequently, failure to detect a change in steady state mRNA does not rule out an increase in ET-1 release in the systemic vasculature. Despite the previous evidence to the contrary, therefore, the hypothesis that ET-1 may mediate the change in systemic vascular endothelial function following exposure to hypoxia is appealing.

ET-1 exerts a prolonged effect once bound to its smooth muscle ET_A receptor. The fact that endothelial ablation of blood vessels resulted in an immediate vasodilation following hypoxia [4,37] suggests that a short-acting constrictor may also be involved. Thromboxane A_2 (TxA_2), is synthesized by the metabolism of prostaglandin H_2 (PGH_2) via thromboxane synthase. TxA_2 has short half-life and, therefore, its more stable metabolite TxB_2 is used as an indirect measure of its production. TxA_2 is released by

endothelial cells in response to stimulation by contractile agonists including ET-1 [169]. Hypoxia directly elicits endothelial release of TxA_2 in some vascular beds and ET-1-induced release of TxA_2 has been shown to increase the vasoconstrictor effect of ET-1 in post-ischemic reperfused rat hearts [170]. It would be interesting to assess whether this scenario of increased ET-1 and TxA_2 occurs in the systemic vasculature and contributes to the observed endothelial enhancement of contraction to adrenoreceptor stimulation.

1.10 Summary

We have previously demonstrated that prolonged hypoxia causes a reversal in endothelial function that involves a loss of its normal inhibitory influence on vascular tone and the activation of mechanisms that enhance agonist-induced contraction. This may be an adaptive mechanism to counteract the reduced smooth muscle contractility following prolonged exposure to hypoxia. It may be accounted for by an increased release in endothelium-derived vasoconstrictors and a decrease in endothelium-derived vasodilator release that would unmask the basal constriction. The most potent endothelium-derived vasodilator and vasoconstrictor are eNOS-derived NO and ET-1, respectively. There is strong evidence indicating that these endothelium-derived substances are under the regulatory influence of oxygen tension and that their expressions are reciprocally regulated. A decrease in eNOS expression and a concomitant increase in ET-1 expression in the aorta would account for the previously observed endothelial enhancement of contraction to adrenoceptor stimulation following hypoxia. These hypotheses will be tested in chapters 2 and 3.

At the level of the smooth muscle, hypoxia has been shown to impair contractile responses to adrenoreceptor stimulation. Interestingly, this effect was shown to be endothelium-independent. The expression of the iNOS has been previously reported to cause smooth muscle hypocontractility in certain disease conditions. Both the iNOS and nNOS have been shown to be hypoxia inducible and in certain conditions can be expressed in vascular smooth muscle. We believe that either or both of these NOS isoforms may be expressed in vascular smooth muscle following hypoxia and contribute

to the observed loss of smooth muscle contractility following hypoxia. This hypothesis will be tested in chapter 4.

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Chapter 2

Downregulation of Endothelial Nitric Oxide Synthase in Rat Aorta Following Prolonged Hypoxia *In Vivo*

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2.1 Foreword

We have previously shown that in both diaphragmatic arterioles [1] and aorta [2], the exposure of rats to hypoxia for 12 and 48 hours *in vivo* causes prominent alterations in both endothelial and smooth muscle cell functions. At the level of the endothelial cell, hypoxia induces a reversal in function such that the vascular endothelium enhances agonist-induced contractions. We hypothesized that this endothelial abnormality may be partly accounted for by a decrease in the production and release of an important endothelium-derived relaxing factor, namely nitric oxide (NO). This potent dilator is synthesized by the Ca^{2+}-dependent endothelial NO synthase (eNOS) and can freely diffuse into the underlying smooth muscle to cause vasorelaxation. Interestingly, the expression and activity of eNOS appear to be under the regulatory control of oxygen. Previous studies on the effect of hypoxia on eNOS expression, however, have been done in cultured endothelial cells from diverse tissues and have yielded conflicting results [3-5]. This chapter addresses the first objectives of this thesis: 1) to determine whether or not the expression of eNOS is downregulated by the exposure to prolonged hypoxia *in vivo*, and 2) to identify other possible alterations in the NO/cGMP pathway which may contribute to the observed endothelium-dependent enhancement of contractility following exposure to hypoxia.

2.2 Abstract

The goal of this study was to determine if hypoxia alters expression of eNOS in the systemic circulation. Rats breathed either air or 10% oxygen for 12 hours, 48 hours or 7 days. Thoracic aorta was excised and either mounted in organ bath myographs or frozen in liquid nitrogen for later extraction of protein and RNA. eNOS protein (Western blotting) was decreased (20% of normoxic control) after 12 hours, 48 hours and 7 days of hypoxia. eNOS mRNA (ribonuclease protection assay) was similarly reduced. Acetylcholine (10^{-4}mol/L) reversed phenylephrine (10^{-5} mol/L) preconstriction by 53.3 ± 5.6% in aortic rings from normoxic rats and 26.1 ± 4.8 % in rings from rats exposed to hypoxia for 48 hours ($p<0.05$) with comparable impairment of relaxation by the calcium ionophore A23187 (10^{-5}mol/L). Responses to diethylamine nitric oxide and 8-bromo-cyclic GMP were unaffected. Aortic cyclic GMP levels following incubation with acetylcholine (10^{-6} mol/L) averaged 14.0 ± 1.8 *f*mol/mg in rings from normoxic rats compared with 8.7 ± 1.0 *f*mol/mg in rings from hypoxic rats ($p<0.05$). Similarly, nitrate concentration (by capillary electrophoresis) in the media in which the rings were incubated was reduced in the hypoxic group (5.6 ± 0.23 μmol/L) compared to 7.8 ± 0.7 μmol/L in normoxic rats. Impaired endothelial NO release may handicap the vascular responses which defend vital organ function during hypoxia.

2.3 Introduction

Hypoxia occurs commonly in patients with cardiopulmonary diseases and in normal individuals at high altitude. Survival under these conditions requires adaptive responses from the systemic circulation which redistribute the available oxygen supply toward vital organs [6] and enhance oxygen extraction [7]. Many of the mechanisms, which mediate these responses, are localized to the vascular endothelium. In particular, endothelial release of nitric oxide (NO), has been shown to modulate myogenic autoregulatory responses [8] and to mediate flow-dilation of resistance arterioles [9] active hyperemia [10], reactive hyperemia [11] and, in some vascular beds, hypoxic vasodilation [12]. Thus, a central role is emerging for endothelial production of NO in maintaining the balance between tissue oxygen supply and metabolic demand. If expression of endothelial nitric oxide synthase (eNOS), the enzyme which catalyses NO synthesis, is regulated by oxygen tension, therefore, changes in the capacity for endothelial NO release may either be an important adaptive response or else contribute to the pathogenesis of vital organ failure depending on whether it is enhanced or impaired, respectively.

The effect of hypoxic incubation on eNOS expression has been investigated previously in endothelial cells in culture [3-5]. Unfortunately the results of these studies have varied in both direction and magnitude. Despite its fundamental clinical and physiological relevance, therefore, the question of whether or not a change in eNOS availability alters vasoregulatory responses during hypoxia remains unanswered. Accordingly, the current study was undertaken to determine if exposure to hypoxia *in vivo* alters expression of eNOS protein and mRNA in the systemic vasculature and to evaluate the effect of this change on endothelium-dependent vasorelaxation.

2.4 Methods

Studies were carried out in adult male Sprague-Dawley rats (200-250 g). All experimental protocols were performed in accordance with institutional guidelines.

2.4.1 Exposure to Hypoxia *In Vivo*

Rats were placed in a sealed Plexiglas chamber (30 cm × 18 cm × 15 cm). Gas flow through the chamber was 6 L/minutes. In animals exposed to hypoxia, the composition of the gas was 10% O_2, 90% N_2. Normoxic animals were exposed to air. At the end of the exposure period (12 hours, 48 hours or 7 days) the thoracic aorta and lungs were excised immediately after decapitation and frozen in liquid nitrogen for later extraction of protein or RNA. In separate experiments, thoracic aorta from normoxic rats and rats exposed to hypoxia for 48 hours were cut into segments and mounted in organ bath myographs for pharmacological studies.

2.4.2 Western Blot Analysis

The thoracic aorta and lungs from normoxic rats (n=4) and from rats exposed to hypoxia for 12 hours (n=4), 48 hours (n=4) or 7 days (n=4) were homogenized in extraction buffer (Tris-HCl 50 mmol/L; glycerol 5%; dithiothreitol 0.1mmol/L; phenylmethylsulfonyl fluoride 100 μg/μL; aprotinin 5.0 μg/mL; leupeptin 5.0 μg/mL; pepstatin 5.0 μg/mL; and trypsin-chymotrypsin inhibitor 5.0 μg/mL, pH 7.4). Proteins were separated on a 4-12% SDS-polyacrylamide gel, transferred onto nitrocellulose by electroblotting and probed with an eNOS specific monoclonal antibody (Transduction Laboratories). Blots were developed using enhanced chemiluminescence. Four Western

blots were carried out using proteins from individual rats from each group (normoxic, hypoxic 12 hrs, hypoxic 48 hrs, and hypoxic 7 days). Bands were quantified by densitometry. Staining with Ponceau Red confirmed the electrotransfer efficiency and was used as an indirect control for loading.

2.4.3 Analysis of eNOS mRNA

The complete coding sequence of the rat eNOS is unknown. A partial length cDNA encoding the C-terminal FAD binding region has previously been cloned [13]; however this region is conserved among the neuronal and inducible nitric oxide synthase isoforms as well as P450 reductases. In order to provide a specific and quantitative assay of eNOS mRNA, we cloned a second partial length cDNA corresponding to the region of least homology with the other isoforms. A cRNA probe transcribed from this cDNA was then used to assess eNOS mRNA levels by ribonuclease protection assay.

RNA extraction and cDNA cloning. The thoracic aorta from normoxic rats (n=5), rats exposed to hypoxia for 12 hours (n=5), 48 hours (n=5) and 7 days (n=5) were excised and frozen in liquid nitrogen immediately following decapitation. Total RNA was extracted using TRIzol™ (Gibco BRL Life Technologies, Gaithersburg, MD) according to the method of Chomczynski and Sacchi [14].

RNA extracted from the thoracic aorta of a rat in the control group was used to clone a partial cDNA fragment of eNOS and β-actin mRNA. One μg of total RNA was denatured by heating at 72°C for 2 minutes and converted to cDNA using M-MLV Reverse Transcriptase (10 U/μL) in a reaction buffer consisting of Tris-HCl (20 mM, pH

8.4), KCl (50 mM), $MgCl_2$ (5 mM), dNTPs (1 mM), RNAase inhibitor (0.16 U/μL), $pd(N)_6$ random primers (5.0 μM).

eNOS cDNA plasmid construct. A 871bp partial cDNA fragment was generated by PCR (30 cycles) from 200 ng total cDNA using eNOS specific sense (5'-AGCTGGCATGGGCAACTTGAA-3') and antisense (5'-CAGCACATCAAAGCGG CCATT-3') primers. These primers were constructed based on the murine eNOS sequence[15] after comparing with those of the murine neuronal [16] and inducible [17] nitric oxide synthase isoforms for regions of least homology. The cDNA amplified using these primers was subcloned into the EcoR1 site of pCR II vector (Invitrogen, San Diego, CA). DNA sequencing indicated 95% homology between this fragment and the murine eNOS mRNA sequence [15] and confirmed its reverse orientation behind the T7 promoter within the vector. The full sequence of this cDNA has been submitted to Genbank (accession number AF085195).

β-actin cDNA plasmid construct. A 292 bp β-actin cDNA was also amplified by PCR (25 cycles) from 5 ng of total cDNA using β-actin specific sense primer (5'-AAGT-ACCCCATTGAACACGGCA-3') and antisense primer (5'-TAGATGGGCAC-AGTGTGGGTGA-3') and subcloned into the EcoR1 site of PCR II vector (Invitrogen, San Diego, CA). DNA sequencing indicated that this cDNA corresponds to nucleotides spanning exons 3 and 4 of the rat β-actin gene [18] and confirmed its reverse orientation behind the Sp6 promoter within the vector. The full sequence of this cDNA has been submitted to Genbank (accession number AF122902).

2.4.4 [^{32}P]-Labeled Riboprobe Synthesis and Ribonuclease Protection Assay

[^{32}P]-riboprobe synthesis. The eNOS construct was linearized by cleavage at position 375 downstream of the T7 promoter with BamHI (4 U/μg plasmid DNA. New England Biolabs, Mississauga, Ontario) while the β-actin construct was linearized with Bsu36I at position 230 downstream of the Sp6 promoter. Antisense RNA for eNOS and β-actin were synthesized using T7 RNA polymerase (20U/μL) and Sp6 RNA polymerase (20U/μL), respectively, along with 1μg of linearized construct and 50 μCi of α-[^{32}P] labeled CTP (400 Ci/mmol, Amersham, UK). The RNA polymerases, nucleotides and transcription buffer were provided with the Riboprobe^{R} *In vitro* Transcription Systems purchased from Promega (Madison, WI). Plasmid DNA was digested with RNAase free DNAase (0.1U/μL) for 30 minutes at 37°C. 20 μg of yeast transfer RNA (Boerhinger-Mannheim, Laval, Quebec) was added as a carrier. The mixture was then extracted with phenol/chloroform/isoamyl alcohol and precipitated twice with ethanol. The RNA pellet was washed with 75% ethanol and resuspended in 500 μL of hybridization buffer consisting of 80% formamide, 0.4 mol/L NaCl, 1 mmol/L EDTA and 40 mmol/L PIPES (pH 6.2). The resulting purified eNOS cRNA probe (375 bases), included 303 bases spanning regions of the rat eNOS mRNA homologous to exons 4, 5, 6 and 7 of the human eNOS sequence [19].

Ribonuclease protection assay. The ribonuclease (RNAase) protection assays were performed on RNA from the thoracic aorta of normoxic rats (n=5), rats exposed to hypoxia for 12 hours (n=5), 48 hours (n=5) and 7 days (n=5). RNAase protection assays for eNOS and β-actin were carried out simultaneously, in the same tube, on each individual aortic RNA sample from each of the 5 rats in each group. Each reaction tube

contained [^{32}P]-labeled eNOS (50,000 cpm) and β-actin (1000 cpm) riboprobes as well as 10 μg of total aortic RNA or yeast transfer RNA. The samples were incubated at 85°C for 5 minutes to denature the RNA and hybridization was carried out overnight at 50°C. 350 μL of RNAase digestion buffer consisting of 10 mM Tris-HCl (pH 7.5), 300 mM NaCl, 5 mM EDTA, 0.04 μg/μL RNAase A and 0.65U/μL RNAase T1 was added to each sample and incubated for 1 hour at 30°C. The samples were incubated 15 minutes at 37°C in the presence of 0.13μg/μL proteinase K and 0.05% SDS. The samples were then extracted with phenol/chloroform/isoamyl alcohol, 10 μg of yeast transfer RNA was added and the mixture was precipitated with ethanol. The pellets were resuspended in RNA loading buffer consisting of 80% (v/v) formamide, 1mM EDTA pH 8.0, 0.1% bromophenol blue and 0.1% Xylene Cyanol and 1X TBE, denatured for 3 minutes at 85°C and run on a 6% polyacrylamide 7M urea gel. The gel was then dried under vacuum and exposed to a PhosphorImager screen (FUJI, BAS-III) overnight. Protected eNOS and β-actin mRNA were quantified by densitometry in each rat aortic RNA sample and the level of eNOS mRNA in each sample was expressed as a percentage of the β-actin mRNA level within the same sample.

2.4.5 Endothelium-Dependent and -Independent Relaxation

Aortic segments (4 mm) from 7 normoxic rats and 7 rats exposed to hypoxia for 48 hours were mounted on stainless steel hooks connected to force/displacement transducers (Grass FT103) in organ baths containing modified Kreb's solution (in mmol/L: Na^+ 143.0; K^+ 5.9; Ca^{2+} 2.5; Mg^{2+} 1.2; Cl^- 151.3; HCO_3^- 25.0; SO_4^{2-} 1.2; $H_2PO_4^-$ 1.4; dextrose 10), bubbled with 95% O_2, 5% CO_2 (pH = 7.4) at 37°C. Tension was

adjusted to 2 g over 1 hour during which the buffer was replaced every 20 minutes. The rings were then contracted with 10^{-5}M phenylephrine and the endothelium-dependent vasodilator acetylcholine (Ach, 10^{-9} mol/L to 10^{-4} mol/L) was administered in a cumulative fashion. The rings were dried at 50°C overnight and weighed so that phenylephrine-activated tension could be expressed in g/mg dry weight. The response to Ach was expressed as percent reversal of the phenylephrine induced contraction. Values obtained in rings from each rat were averaged and the averaged values were counted as single observations. Concentration-response relationships were evaluated by comparing the maximum Ach-induced relaxation and the concentration that produced a 50% maximal response (EC_{50}).

Separate groups of aortic rings (4 mm) from 7 normoxic rats and 7 rats exposed to hypoxia for 48 hours were equilibrated to a baseline tension of 2 g then precontracted with phenylephrine (10^{-5} mol/L) as above. The response to Ach (10^{-5} mol/L) was assessed in each ring. The rings were then washed to remove these drugs, allowed to re-equilibrate to their previous baseline tension of 2g and again contracted with phenylephrine (10^{-5} mol/L). One of either A23187 (calcium ionophore, 10^{-5} mol/L), diethylamine nitric oxide (DEA/NO, NO donor, 10^{-4} mol/L) or 8-Bromo-cyclic guanosine monophosphate (8-Br-cGMP, cell permeable cGMP, 10^{-4} mol/L) was then added to the organ baths. Values for each agent obtained in rings from a given rat were averaged and the averaged values counted as single observations.

2.4.6 Cyclic GMP Radioimmunoassay

Thoracic aorta from normoxic rats (n=6) and rats exposed to hypoxia for 48 hours (n=9) were cut into 2 mm segments which were randomly allocated to one of 3 groups. All were equilibrated for 1 hour in 4 mL of Kreb's solution, pH 7.4, at 37°C then incubated for 10 minutes in isobutylmethylxanthine (0.5 mmol/L). In two of the groups of segments, Ach (10^{-6} mol/L) was then added. One group was incubated with Ach for 1 minute and one group was incubated for 4 minutes. In the third group no Ach was added. At the end of the exposure period the reaction was stopped by the addition of trichloroacetic acid (TCA, final concentration = 10%) to the bath. The reaction buffer from rings not exposed to Ach and from rings exposed to Ach for 4 minutes was stored for measurement of nitrate concentration by capillary electrophoresis (see below).

The aortic segments were weighed and homogenized in 1 mL 10% TCA. The samples were centrifuged at 14,000 × g for 20 minutes and the supernatants removed. TCA was extracted from the supernatants by four consecutive washes with water saturated ether. Residual ether was evaporated by aeration and the samples were acetylated at room temperature using 25 µL of a 2:1 mixture of trifluoroethane and glacial acetic acid. The reaction was stopped with acetate buffer pH 6.6 consisting of CH_3COONa (0.35 mol/L), NaCl (0.15 mol/L), 2% BSA and 0.1% NaN_3. Rabbit anti-rat cGMP (1:20,000) was added to 400 µL of each sample and incubated for 48 hours at 4°C. 100 µL of ^{125}I labeled cGMP (10,000 cpm) was then added and the mixture was incubated at 4°C for an additional 24 hours. Finally the samples were incubated for 2 hours at room temperature with 100 µL of 1:50 normal rabbit serum and 100 µL of 1:25 IgG and precipitated with 12.5% polyethylene glycol. Following centrifugation, the

supernatants were removed and the radioactivity in the pellets was measured using a gamma counter. Values obtained for each period of Ach incubation in segments from a given rat were averaged and the averaged values counted as single observations.

2.4.7 Quantification of Nitrate by Capillary Electrophoresis

Plasma nitrate ion (NO_3^-) concentration was measured by capillary electrophoresis (CE) using a modification of previously described techniques [20,21]. Immediately following decapitation, blood was collected from normoxic rats (n=6) and from rats exposed to hypoxia for 48 hours (n=6) in 10 mL Vacutainer™ tubes. The samples were spun at 2,000 × g for 10 minutes at 4°C. The plasma was carefully removed and centrifuged at 12,000 × g for an additional 10 minutes at 4°C. The plasma was then passed through a 0.2 μm sterile membrane filter and analyzed by an ABI 270 CE instrument with a fused silica capillary (365 μm outside diameter, 50 μm internal diameter (Polymicro Technologies, Phoenix, AZ). Nitrate analysis was performed in a buffer containing 50 mmol/L phosphate pH 2.5, supplemented with 0.5 mmol/L spermine as an electro-osmotic flow modifier. Samples were loaded into the capillary by automatic injection under a vacuum of 17 kPa for 1.5 seconds and the separation was carried out at an applied electric field of -347 V cm^{-1} (current = 45-50 μA). These parameters allowed the detection of NO_3^- peak by ultraviolet absorption at 214 nm. This wavelength was chosen because chloride, which normally migrates in close proximity to NO_3^- and partially obscures its peak, does not absorb at 214 nm. The capillary oven temperature was at 30°C during the analysis. All buffers were made fresh daily in doubly deionized water (Milli-Q unit; Millipore, Montreal, PQ, Canada) and filtered through 0.45 μm

membrane filters (Gelman Sciences, Montreal, PQ, Canada) before use. Triplicate runs were carried out for each sample and the mean value was used in the analysis. Following each run, the capillary was washed with 0.5 mol/L NaOH (2 min.), with double deionized water (2 min.) and finally with running buffer (6 min.). Data were collected with an integrator Model SP4600 (Spectra-Physics, San Jose, CA) and Spectra-Physics Winner software was used for data storage and analysis. Peak areas were normalized for the time of NO_3^- migration and used for quantification. Calibration curves were generated using plasma spiked with standard nitrate solutions and were linear (r=0.998) over the 31-1010 μmol/L range.

The above CE parameters were also used to measure the concentration of NO_3^- in the reaction media from unstimulated aortic segments and from aortic segments that were incubated with Ach (10^{-6} mol/L) for 4 minutes (see above). Calibration curves were constructed with reaction media spiked with standard nitrate solutions (final concentrations 1.92-55.6 μmol/L) and were linear (r=0.996) over this range.

2.4.8 Statistical Analysis

Comparisons between groups were performed by analysis of variance (ANOVA). If the ANOVA revealed significant overall differences, variations among individual means were evaluated post-hoc using the Student-Neuman-Keuls procedure. Results are expressed as the means ± SEM for aortic segments from *n* number of animals, with $p < 0.05$ representing significance.

2.5 Results

2.5.1 eNOS Protein Expression

Representative Western blots conducted on proteins from thoracic aorta and lungs from a normoxic rat and rats exposed to hypoxia for 12 hours, 48 hours and 7 days are illustrated in Figure 2.1. The accompanying histograms illustrate the mean aortic and lung eNOS protein levels (arbitrary units of optical density) for each group (n=4 per group). Aortic eNOS protein was decreased ($p<0.01$ vs. normoxic controls) following 12 hours, 48 hours and 7 days of hypoxia. Lung eNOS protein levels were unchanged following 12 and 48 hours of hypoxia but were increased ($p<0.05$ vs. normoxic controls) following 7 days of hypoxia as previously reported [22-24].

2.5.2 eNOS mRNA Expression

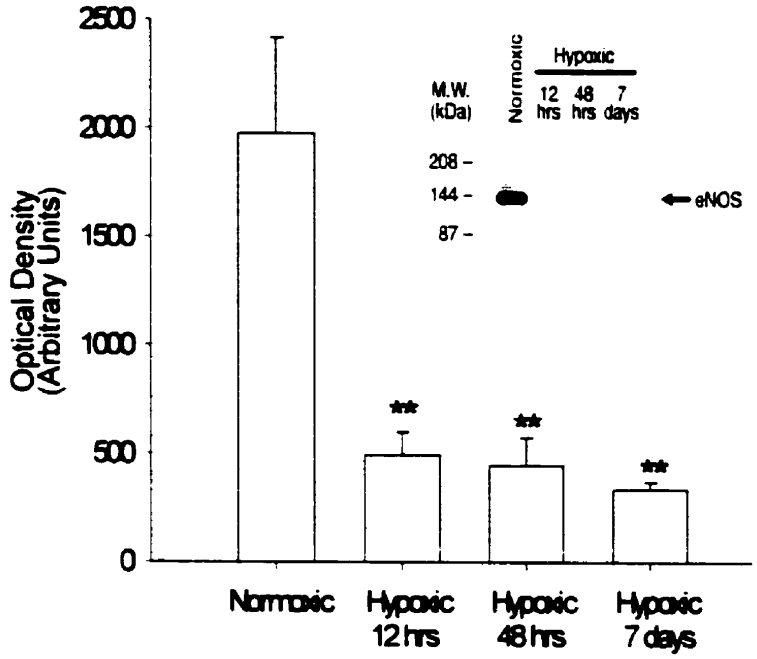
A representative autoradiogram of an RNAase protection assay carried out on aortic RNA from a normoxic rat and from rats exposed to hypoxia for 12 hours, 48 hours and 7 days is illustrated in Figure 2.2A. The histogram in Figure 2.2B illustrates the mean aortic eNOS mRNA levels in each group (n=5 per group) expressed as percent of the β-actin mRNA level. Aortic eNOS mRNA levels are decreased ($p<0.01$) following exposure to hypoxia for 12 hours, 48 hours and 7 days compared with normoxic controls.

Figure 2.1

eNOS Protein Expression in Rat Aorta and Lung

Representative Western blots of endothelial nitric oxide synthase (eNOS) carried out on proteins from thoracic aorta (**A**) and lungs (**B**) of a normoxic rat, and from rats exposed to hypoxia for 12 hours, 48 hours and 7 days. Histograms illustrate the mean aortic (**A**) and lung (**B**) eNOS protein levels (arbitrary units of optical density) for each group ($n = 4$ per group). *, $p < 0.05$ vs. normoxic controls. **, $p < 0.01$ vs. normoxic controls.

A


Optical Density (Arbitrary Units)
2500
2000
1500
1000
500
0
M.W. (kDa)
Normoxic
Hypoxic
12 hrs
48 hrs
7 days
208 –
144 –
87 –
eNOS
**
**
**
Normoxic
Hypoxic 12 hrs
Hypoxic 48 hrs
Hypoxic 7 days

B

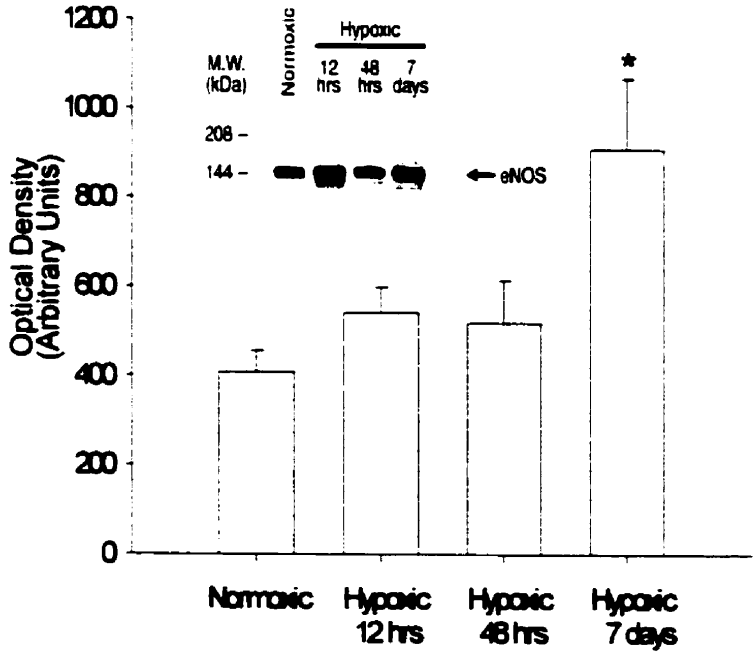
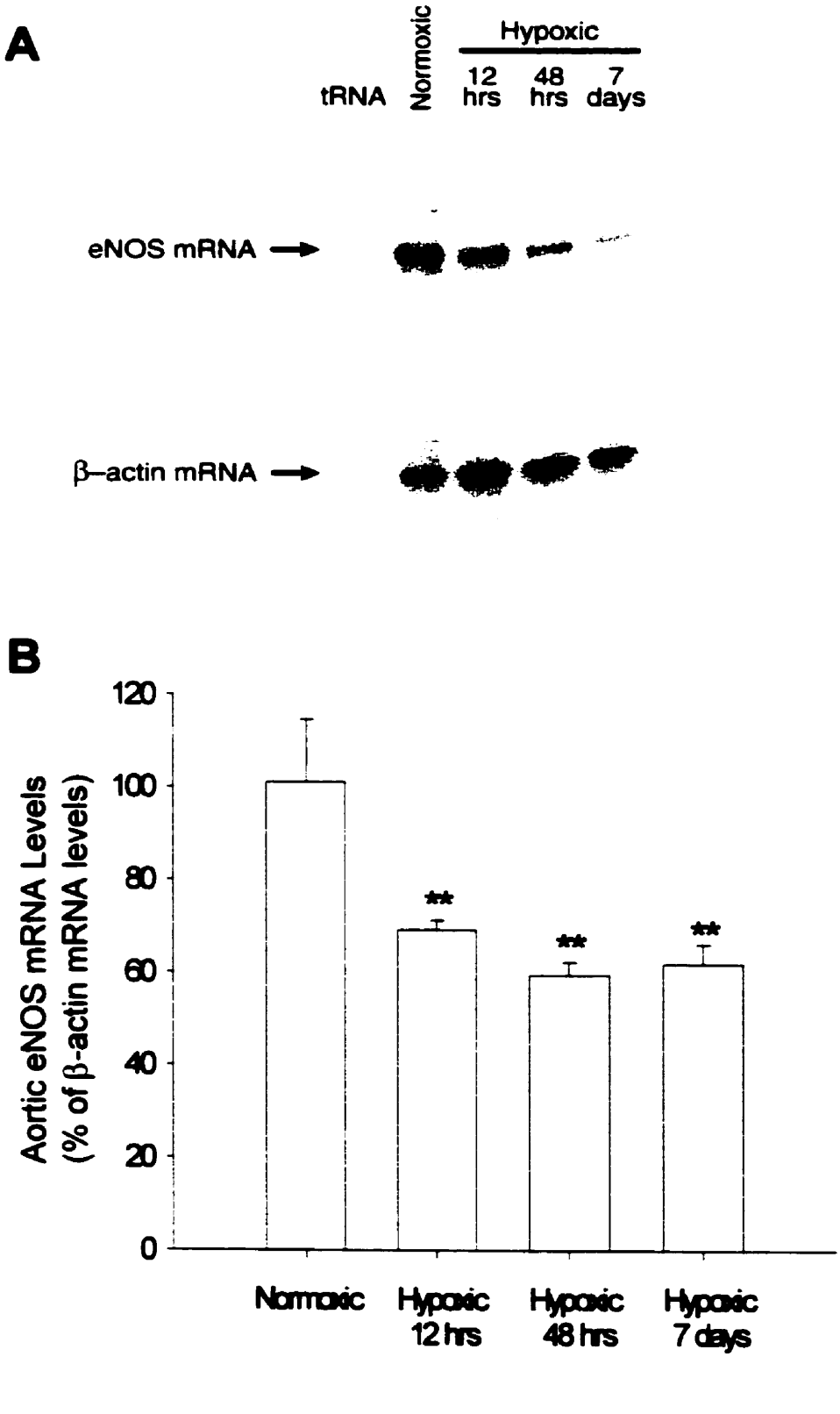

Optical Density (Arbitrary Units)
1200
1000
800
600
400
200
0
M.W. (kDa)
Normoxic
Hypoxic
12 hrs
48 hrs
7 days
208 –
144 –
eNOS
*
Normoxic
Hypoxic 12 hrs
Hypoxic 48 hrs
Hypoxic 7 days

Figure 2.2

eNOS mRNA Expression in Rat Aorta

A, Representative RNAase protection assay carried out on aortic RNA from a normoxic rat and from rats exposed to hypoxia for 12 hours, 48 hours and 7 days. **B**, Histogram illustrates mean aortic eNOS mRNA levels in each group (n=5 per group) expressed as percent of the β-actin mRNA level. **, $p<0.01$ vs. normoxic controls.


A
Normoxic
Hypoxic
12 hrs
48 hrs
7 days
tRNA
eNOS mRNA
β–actin mRNA
B
Aortic eNOS mRNA Levels
(% of β-actin mRNA levels)
120
100
80
60
40
20
0
**
**
**
Normoxic
Hypoxic 12 hrs
Hypoxic 48 hrs
Hypoxic 7 days

2.5.3 Endothelium-Dependent and -Independent Relaxation

The concentration-response relationships for acetylcholine induced relaxation in aortic rings from normoxic rats and from rats exposed to hypoxia for 48 hours are illustrated in Figure 2.3A. The tension generated during contraction with 10^{-5} mol/L phenylephrine was 1.67 ± 0.08 g/mg dry weight in rings from normoxic rats. As has been reported previously [23], tension was lower (0.88 ± 0.06 g/mg dry weight) in rings from hypoxic rats. Maximal relaxation by Ach was 53.3 ± 5.6 % of the phenylephrine-induced contraction in rings from normoxic rats, compared to 26.1 ± 4.8 % in rings from rats exposed to hypoxia ($p < 0.05$ for difference). The pEC_{50} for Ach induced relaxation was 6.90 ± 0.18 in rings from normoxic rats and 7.21 ± 0.14 in rings from hypoxic rats ($p > 0.05$).

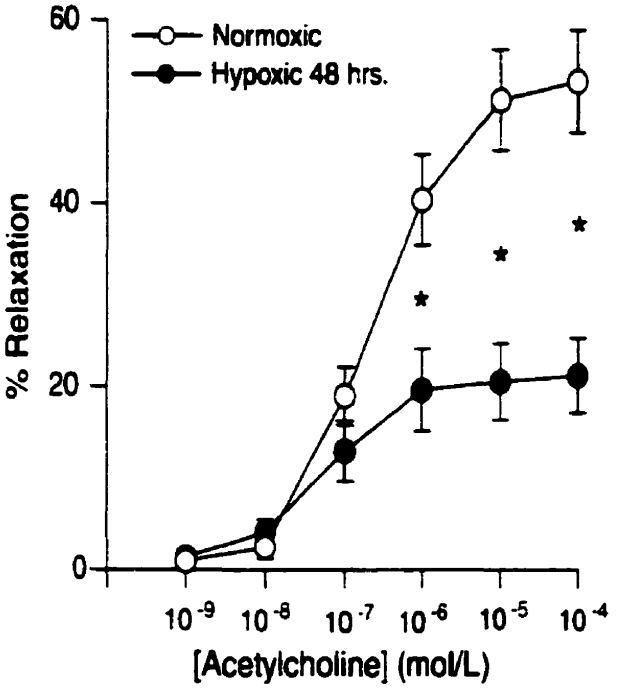
The responses to A23187, DEA/NO and 8-Br-cGMP in aortic rings from normoxic rats and from rats exposed to hypoxia for 48 hours are compared in Figure 2.3B. Relaxation of phenylephrine contraction by A23187 was reduced in the hypoxic group. The responses to DEA/NO and 8-Br-cGMP in aortic rings from hypoxic rats did not differ from those in rings from normoxic rats. In this group, acetylcholine reversed 48.5 ± 3.8 % of the phenylephrine-induced contraction in rings from normoxic rats, and 20.1 ± 2.8 % in rings from rats exposed to hypoxia ($p < 0.05$ for difference).

Figure 2.3

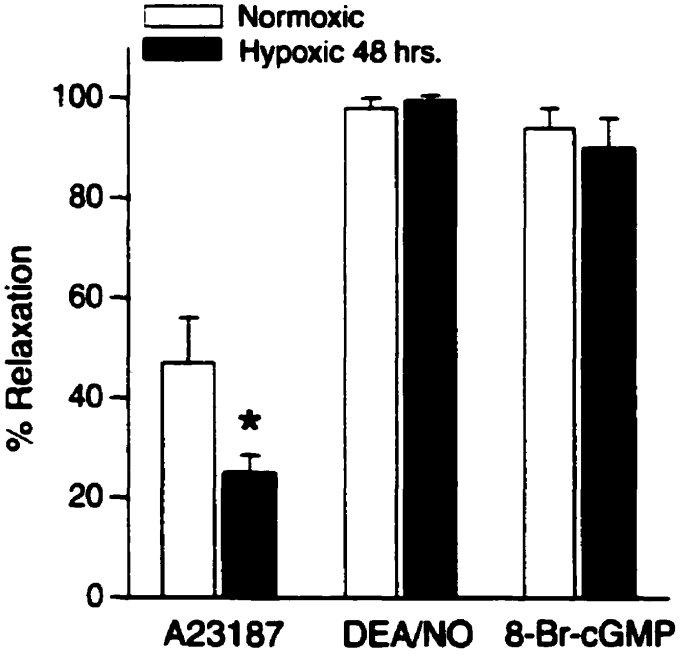
Endothelium-Dependent and -Independent Relaxation

A, Concentration response relationships for ACh-induced relaxation of phenylephrine-pre-constricted aortic rings from normoxic rats and rats exposed to hypoxia for 48 hours. *, $p < 0.05$ for difference between normoxic and hypoxic groups. **B**, Relaxation by A23187 (10^{-5} mol/L), diethylamine nitric oxide (DEA/NO, 10^{-4} mol/L), and 8-bromo-cGMP (10^{-4} mol/L) of phenylephrine-preconstricted aortic rings from normoxic rats and rats exposed to hypoxia for 48 hours. *, $p < 0.05$ for difference between normoxic and hypoxic groups.

A


Normoxic
Hypoxic 48 hrs.
% Relaxation
60
40
20
0
*
*
*
10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}
[Acetylcholine] (mol/L)

B


Normoxic
Hypoxic 48 hrs.
% Relaxation
100
80
60
40
20
0
*
A23187
DEA/NO
8-Br-cGMP

2.5.4 cGMP Generation

The effect of hypoxia on aortic cGMP levels during *in vitro* stimulation with acetylcholine is illustrated in Figure 2.4. Unstimulated cGMP levels did not differ between normoxic rats and those exposed to hypoxia for 48 hours. The mean aortic cGMP level after 4 minutes of incubation with acetylcholine was lower ($p < 0.05$) in the group exposed to hypoxia for 48 hours than in the normoxic group.

2.5.5 Plasma Nitrate Concentration and Aortic Nitrate Production

Concentrations of NO_3^- in plasma from normoxic rats (n=7) and from rats exposed to hypoxia for 48 hours (n=6) averaged 70.3 ± 2.6 μmol/L and 72.1 ± 5.2 μmol/L, respectively ($p > 0.05$ for difference). The concentrations of nitrate in the reaction buffer from aortic rings from normoxic rats and from rats exposed to hypoxia for 48 hours which were not treated with Ach (control) and which were incubated with 10^{-6} mol/L Ach for 4 minutes are illustrated in Figure 2.5. Unstimulated values in the two groups did not differ. Nitrate concentrations were higher ($p < 0.05$) following incubation with Ach in the normoxic than in the hypoxic group.

Figure 2.4

cGMP Levels in Rat Aorta

cGMP levels in aortic segments from normoxic rats and rats exposed to hypoxia for 48 hours following incubation with acetylcholine for 0, 1 and 4 minutes. * $p < 0.05$ for difference between normoxic and hypoxic groups. Data are mean ± SEM.

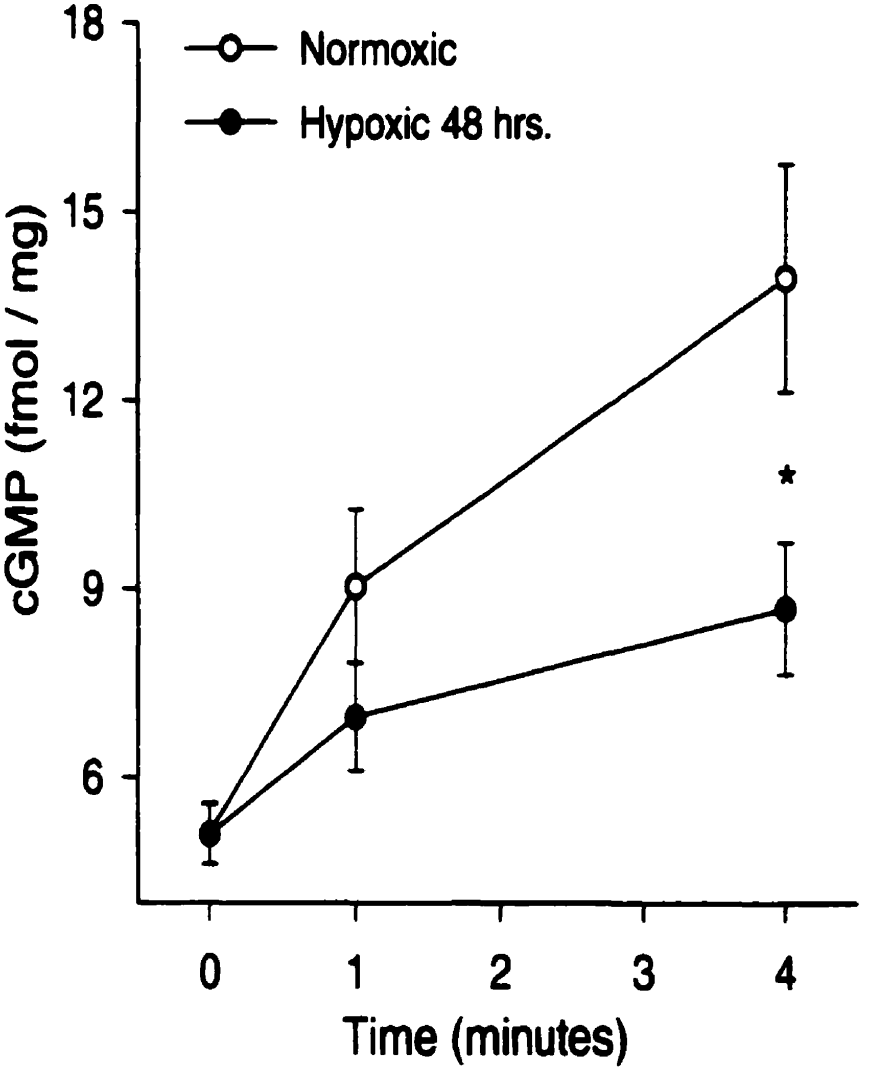
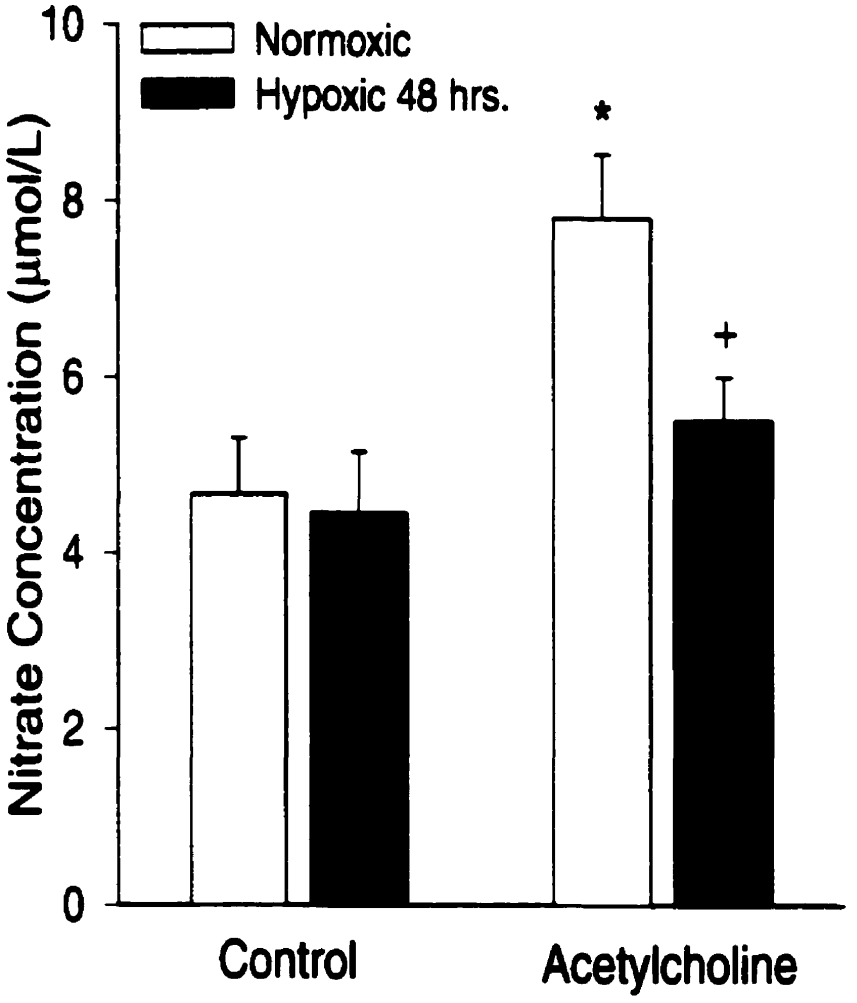

Normoxic
Hypoxic 48 hrs.
cGMP (fmol / mg)
18
15
12
9
6
*
0
1
2
3
4
Time (minutes)

Figure 2.5

Nitrate Concentration in Rat Aorta in Response Acetylcholine

Nitrate concentrations in buffer from aortic rings from normoxic rats and rats exposed to hypoxia for 48 hours incubated in the presence and absence (control) of ACh (10^{-6} mol/L). * $p < 0.05$ for difference from the unstimulated control value. + $p < 0.05$ for difference from corresponding value in the normoxic group. Data are mean ± SEM.


Normoxic
Hypoxic 48 hrs.
Nitrate Concentration (µmol/L)
10
8
6
4
2
0
*
+
Control
Acetylcholine

2.6 Discussion

The results of this study indicate that in rats, prolonged exposure to hypoxia results in: 1) a decrease in aortic eNOS protein and mRNA; 2) impaired endothelium-dependent relaxation of phenylephrine pre-contracted aortic rings; and 3) impaired capacity of aortic segments to generate cGMP and NO_3^- in response to stimulation by acetylcholine. This is the first demonstration that physiologically relevant levels of hypoxia *in vivo* alter eNOS expression in the systemic vasculature and, consequently, impair endothelium-dependent vascular responses. Our finding that, following exposure to hypoxia, relaxation in response to A23187 is decreased to a similar extent as is relaxation to Ach points to an abnormality distal to events occurring at the level of the endothelial cell plasma membrane (i.e. Ach receptor activation and Ca^{2+} entry). The alteration must also be upstream of soluble guanylate cyclase activation or cGMP target sites because the relaxant effects of DEA/NO, and 8-Bromo-cGMP were not affected. The abnormality in endothelium-dependent relaxation following *in vivo* hypoxia, therefore, is not due to the inability to activate eNOS or to respond to its product (NO), but rather to a reduction in the availability of this enzyme.

Previous studies of the effects of hypoxia on eNOS protein and mRNA expression in cultured endothelial cells have yielded conflicting results. Hypoxic incubation (0% O_2 for 24 hours) decreased eNOS protein and mRNA in endothelial cells from human umbilical [3] and saphenous veins [25,26], and from bovine pulmonary artery [4] and aorta [4,26]. In contrast, Arnet and colleagues [5] reported that eNOS protein and mRNA were increased in bovine aortic endothelial cells after 24 hours of incubation at 1% O_2. Moreover, a luciferase reporter construct consisting of the eNOS 5' regulatory region could be

activated in these cells by hypoxia. Upregulation of eNOS protein and activity has also been demonstrated in early passage porcine coronary arteriolar endothelial cells following hypoxic exposures of 30 to 240 minutes duration [27]. This variability reflects differences in the species and vascular bed from which the endothelial cells were derived, the methods used to maintain the cells, and the duration and severity of the hypoxic exposures. Even if the previous data were consistent, however, cell culture experiments may not accurately reproduce the microenvironment to which these cells are normally exposed nor the chemical and mechanical stimuli which interact with the effects of hypoxia under physiological conditions. Convincing evidence that hypoxic regulation of eNOS protein expression is a physiologically relevant mechanism, therefore, requires its demonstration *in vivo* and the present results complement and extend the findings of the previous studies.

The effect of *in vivo* exposure to hypoxia on eNOS protein expression has previously been investigated in the rat pulmonary circulation [22,23,28]. In those studies, breathing 10% oxygen for 7 days and for 3 weeks increased pulmonary eNOS levels. This could not be attributed to the known stimulatory effect of increased flow (shear stress) on eNOS expression [24] because inhibiting the increase in pulmonary blood flow by surgical stenosis of the pulmonary artery failed to prevent the increase in eNOS. Our present results confirm upregulation of lung eNOS protein following 7 days of hypoxia and demonstrate that hypoxia has the opposite effect on eNOS expression in the aorta.

Aortic blood flow is also increased in rats during hypoxia [6]. Although shear stress at the endothelial-luminal interface may not necessarily increase in tandem with flow, it is highly unlikely to change in the opposite direction. Consequently, the decrease in aortic

eNOS that we describe in this report is not likely to be attributable to changes in flow. This study does not include experiments designed to dissociate the influence of other hemodynamic or neurohumoral stimuli from the direct effects of hypoxia. Nonetheless, these factors comprise part of the response to systemic hypoxia and the hypoxic exposure as presented in this study simulate a clinically and physiologically relevant condition. Taken together, our current results and those of previous *in vivo* studies indicate marked regional variability in eNOS protein expression during systemic hypoxia.

The endothelium normally exerts an inhibitory effect on vascular reactivity and agonist- induced contraction is greater in arterial segments from which the endothelium has been removed compared to those in which it is intact [2,29]. In previous studies, we have noted that, after prolonged hypoxic exposure, endothelial ablation resulted in a decrease rather than an increase in the contractile response of rat aortic segments to phenylephrine [2]. Following hypoxia, therefore, the endothelium serves as a source of substances which enhance rather than inhibit contraction. Our current results provide a partial mechanistic explanation for this finding because the inhibitory influence of the endothelium on vascular reactivity has been attributed to endothelial NO release [29]. A decreased capacity for vasodilator synthesis, however, cannot account for endothelial enhancement of contractility and a concomitant increase in endothelium-derived constricting factor release must be proposed. Synthesis and receptor binding of both endothelin-1 [30,31] and thromboxane A_2 [32,33] have been reported to be under the negative regulatory influence of NO. Accordingly, hypoxic inhibition of eNOS expression may play an additional role in the alteration in endothelial function through the removal of an inhibitor of vasoconstrictor production and activity.

Plasma nitrate levels did not differ between normoxic rats and rats exposed to hypoxia for 48 hours in the current study, indicating that factors other than aortic eNOS levels determine the circulating NO_3^- concentration. During hypoxia, increased flow [6] will stimulate NO synthesis by the remaining enzyme [34]. The duration of eNOS activation may be increased due to increased pH in the endothelial intracellular space [35,36] and increased eNOS expression in the lung [28] may counterbalance the effect of decreased NO synthesis in the systemic vasculature. Our inability to demonstrate a decrease in circulating NO_3^-, therefore, does not diminish the pathophysiological significance of hypoxic inhibition of eNOS expression in the systemic circulation. The dilatory response to flow needed to maximize perfusion [9] and the decrease in transvascular resistance necessary to accommodate increased metabolic activity [10] and to preserve vital organ perfusion during superimposed hypotensive stresses [8] require that local NO production be intact. A decrease in the capacity of the endothelium to maximally respond to dilatory stimuli would undermine these responses.

Inhibition of NO synthesis by infusion of L-arginine analogues has been shown to inhibit hypoxic vasodilation in the guinea pig heart [12] and canine diaphragm [37]. In at least some essential vascular beds, therefore, this pathway must be intact in order to optimize perfusion and maintain tissue oxygenation in the face of decreased systemic oxygen delivery. These responses will be impaired by a reduction in the local capacity to produce NO. Since, the decrease in eNOS protein expression occurs relatively quickly (hours to days), impaired endothelial NO release is relevant to cardiopulmonary diseases associated with hypoxia (e.g. pneumonia, congestive heart failure and exacerbations of chronic obstructive lung disease) whose natural histories evolve over this time frame. Hypoxic inhibition of eNOS expression in the systemic circulation may, therefore,

represent an important mechanism in the pathogenesis of organ dysfunction in critically ill patients.

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Chapter 3

Increased Endothelin-1 Expression in Rat Aorta Mediates Endothelial Enhancement of α-Agonist-Induced Contractions Following Hypoxia *In Vivo*

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to *Circulation Research* for publication.

3.1 Foreword

Hypoxia causes a reversal in vascular endothelial function such that the endothelium becomes a net vasoconstrictor and thereby enhances agonist-induced contractions. This may be partly accounted for by our findings in chapter 2 which demonstrate that the endothelial nitric oxide synthase (eNOS) is profoundly downregulated in the systemic vasculature following exposure to physiologically relevant levels of hypoxia *in vivo*. This decrease in the capacity for vasodilator synthesis, alone however, cannot account for the observed endothelial enhancement of contractility. We therefore hypothesized that an increase in endothelium-derived constricting factor release must also occur concomitantly. Endothelin-1 (ET-1) is a very potent endothelium-derived vasoconstrictor[1] and its expression has been shown to be increased in endothelial cells cultured under hypoxic conditions and in the plasma and lung tissue following exposure to hypoxia *in vivo* [2-8]. Surprisingly, however, previous attempts to demonstrate a local increase in ET-1 expression in vascular tissues *in vivo* have not been successful and possibly due to the use of relatively insensitive methods of analysis.

Hypoxia and ET-1 have been shown to induce the endothelial release of the vasoconstrictor, thromboxane A_2 (TxA_2), in some vascular beds and ET-1-induced release of TxA_2 has been shown to increase the vasoconstrictor effect of ET-1 in post-ischemic reperfused rat hearts [9]. Since the synthesis and receptor binding of ET-1 and TxA_2 are under the negative regulatory influence of NO [10-13], the hypoxic inhibition of eNOS expression, demonstrated in the previous chapter, may play an additional role in the alteration of endothelial function through the removal of an inhibitor of vasoconstrictor production and activity. The purpose of the following study is to test the possibility that hypoxia locally increases ET-1 and TxA_2 expression in rat aorta and whether or not these

factors play a role in the observed endothelial enhancement in aortic contractility to adrenoreceptor stimulation following hypoxia.

3.2 Abstract

The purpose of this study was to determine whether or not ET-1 mediates endothelium-dependent enhancement of rat aortic contractility in response to adrenoreceptor stimulation following exposure to hypoxia, and if so, to test the involvement of thromboxane A_2 in this ET-1-mediated effect. Rats breathed room air or 10 % oxygen for 12 or 48 hours. Thoracic aortas were analyzed for ET-1 and thromboxane B_2 content by radioimmunoassay. PreproET-1 mRNA levels were measured by ribonuclease protection assay. Aortic rings (4 mm) were mounted in organ bath myographs for measurement of tension during activation by phenylephrine (PE, 10^{-9}-10^{-5}M) in the presence and absence of: 1) the ET_A receptor-specific antagonist BQ-123 (10^{-7}M); 2) ET-1 at subthreshold concentrations ($3X10^{-10}$M) or those reported in plasma during hypoxia (10^{-12}M, $6X10^{-12}$M); and 3) the thromboxane A_2 / endoperoxidase receptor antagonist, SQ29548 (10^{-7}M). Aortic ET-1 levels were 8.06 ± 0.94 pg/mg in normoxic rats, compared to 11.94 ± 1.84 pg/mg and 13.89 ± 2.03 pg/mg in rats exposed to hypoxia for 12 and 48 hours, respectively ($p< 0.01$, 48 hours versus control) and 5.67± 1.60 pg/mg in aortas from rats exposed to hypoxia for 48 hours from which the endothelium had been removed ($p>0.05$ versus control). Aortic preproET-1 mRNA was increased after 12 and 48 hours of hypoxia compared to the normoxic group. Maximum tension during PE-induced contraction was 1.41 ± 0.06 mg/g and 1.25 ± 0.04 mg/g in endothelialized rings from rats exposed to hypoxia for 48 hours in the presence and absence of BQ123 respectively ($p<0.05$ for difference), and 1.22 ± 0.10 mg/g in rings in which the endothelium had been removed. The addition of ET-1, at subthreshold concentrations or at concentrations measured in plasma of hypoxic rats in the current

study, did not alter the PE response of aortic rings from rats exposed to normoxia or hypoxia. Interestingly, treatment with SQ29548 of aortic rings from normoxic rats decreased the maximum response to PE while it had no effect on rings from the hypoxic group. This hypoxia-induced alteration in the responsiveness of aortic rings from hypoxic rats to TxA_2 / PGH_2 represents a previously unidentified vascular response to hypoxia. Endothelium-derived ET-1 release accounts for the observed endothelial enhancement of contractility following hypoxia, and TxA_2 is not involved in this response. Local ET-1 release is an important compensatory mechanism by which the responsiveness of systemic vasculature is preserved during hypoxia.

3.3 Introduction

Hypoxemia elicits systemic vascular reflexes, mediated primarily by the sympathetic nervous system [14-16], which enhance oxygen extraction and redistribute the available oxygen supply to vital organs. The maintenance of vital organ function, during reduction in systemic oxygen delivery, depends on the degree to which these responses are preserved while the vascular tissues themselves are rendered hypoxic. Systemic vascular reactivity is impaired in chronically hypoxic rats [17] and humans [18] and smooth muscle contractility is diminished in aortic segments [19] and arterioles [20] from rats exposed to hypoxia for 12 to 48 hours. Consequently, the capacity to actively regulate the systemic circulation, and the vascular responses that defend tissue oxygenation, are threatened if oxygen deprivation is prolonged.

We have previously demonstrated that the impairment of arterial smooth muscle contractility following prolonged hypoxia, is compensated by the release of a constricting factor(s) from the vascular endothelium [19,20]. This suggests mechanisms for counteracting hypoxia-induced systemic vascular dysfunction that are localized to the endothelium and warrant further investigation. The potent vasoconstrictor, endothelin, is released from vascular endothelial cells in response to mechanical [21] and chemical [1,22] stimuli. The endothelin-1 (ET-1) isoform, and its mRNA, are increased in endothelial cells cultured under hypoxic conditions[3]. Moreover, plasma ET-1 levels are increased in rats and humans during hypoxia[2,4-8] and reduction in ambient oxygen tension increase ET-1 production in arterial segments *in vitro* [23,24]. Increased endothelium-derived ET-1 release in the systemic vasculature is, therefore, an attractive candidate for mediating endothelium-dependent enhancement of agonist-induced contraction after prolonged exposure to hypoxia in systemic arterial vessels.

In previous studies, an increase in preproET-1 mRNA could not be detected by Northern analysis in the systemic vasculature of rats following exposure to hypoxia, despite an increase in plasma ET-1 concentrations [5,8]. It was concluded that production of ET-1 is not part of the systemic vascular response to physiologically relevant levels of hypoxia *in vivo*. Northern blotting is, however, an insensitive method by which to exclude the expression of a potent vasoconstrictor, which may exert important effects at low concentrations. Furthermore, ET-1 production may be regulated at the level of translation or during post-translational enzymatic processing [25,26] as well as at the level of transcription. Consequently, failure to detect a change in steady state mRNA does not rule out an increase ET-1 release in the systemic vasculature. Despite the previous evidence to the contrary, therefore, the hypothesis that ET-1 mediates the change in systemic vascular endothelial function following exposure to hypoxia remains appealing and requires further specific evaluation.

ET-1 exerts a prolonged effect once bound to its smooth muscle receptor. The fact that endothelial ablation of blood vessels resulted in an immediate vasodilation following hypoxia [19,20] suggests that a short-acting constrictor may also be involved. Thromboxane A_2 (TxA_2), whose short-acting effects are mediated through its receptor on smooth muscle cells, is released by endothelial cells in response to stimulation by contractile agonists including ET-1 [27]. Hypoxia directly elicits endothelial release of TxA_2 in some vascular beds and ET-1-induced release of TxA_2 has been shown to increase the vasoconstrictor effect of ET-1 in postischemic reperfused rat heart [9]. Finally, the synthesis and receptor binding of ET-1 and TxA2 are under the negative regulatory influence of NO. We have previously demonstrated that our model of hypoxia is NOS-deficient and therefore will favor the release and activity of these factors.

The current study was undertaken to determine if ET-1 and TxA_2 are increased in the aorta following prolonged hypoxia and whether or not these vasoconstrictors mediate endothelial enhancement of α-adrenoceptor agonist induced aortic contraction under these conditions.

3.4 Methods

Studies were carried out in adult male Sprague-Dawley rats (200-250 g). All experimental protocols were performed in accordance with institutional guidelines.

3.4.1 Exposure to Hypoxia *In Vivo*

Rats were placed in a sealed Plexiglas chamber (30 cm x 18 cm x 15 cm) and exposed to hypoxia for 12 or 48 hours. All rats were provided with rat chow and water *ad libitum*. The inflow of air and nitrogen into the chamber were controlled independently and the outflow was through an underwater seal. The gas inflow consisted of air at a rate of 3 L/min. and nitrogen at 3 L/min. (inspired O_2 concentration = 0.1) which resulted in a total flow rate of 6 L/min. and prevented CO_2 accumulation. Normoxic rats were exposed to air only. Gas samples were drawn periodically from the chamber for analysis (AVL Instruments, Graz Austria, model 995) to ensure maintenance of the appropriate ambient PO_2. The temperature within the chamber was monitored using a temperature probe (Physitemp Instruments Inc., Clifton NJ, SST1) and remained the same as the surrounding room temperature throughout the exposure period. In preliminary experiments (n=4) in which blood was sampled from a cannula in the carotid artery, the arterial PO_2 averaged 38 torr (range 35-42) and the arterial PCO_2 was 32 torr (range 29-34). Rats were sacrificed by decapitation and the thoracic aorta was immediately used for pharmacological studies, or frozen in liquid nitrogen for biochemical studies of ET-1 and TxA_2.

3.4.2 Aortic Contractility

The thoracic aorta from normoxic rats and from rats exposed to hypoxia for 48 hours was removed immediately after decapitation, cleaned of connective tissue, and cut

into segments of 4 mm in length. The segments were then either denuded of their endothelium with a wooden spatula or left intact, and mounted on stainless steel hooks in 15 mL jacketed organ baths containing modified Krebs solution (in mM: Na^+ 143.0; K^+ 5.9; Ca^{2+} 2.5; Mg^{2+} 1.2; Cl^- 153.9; HCO_3^- 25.0; SO_4^{2-} 1.2; $H_2PO_4^-$ 1.2; dextrose 10.0). The hooks were connected to Grass force/displacement transducers (model FT03) and tension was gradually adjusted to 2 grams over 1 hour. The solution in the bath was kept at 37°C and bubbled with a 95% O_2, 5% CO_2 gas mixture. To ascertain the integrity of the endothelium, the response to the endothelium-dependent vasodilator acetylcholine (ACh, 10^{-4} M) was assessed after contraction with phenylephrine (PE, 10^{-5}M). All endothelium-intact aortic rings demonstrated relaxation of PE-induced contraction upon addition of ACh to the bath. In rings in which the endothelium had been removed, the absence of vasorelaxation in response to ACh was taken as evidence that endothelial ablation had been successful. Changing the solution in the organ bath then washed out phenylephrine and ACh and tension was allowed to return to its previous baseline value of 2 grams. In endothelium-intact and endothelium-denuded aortic rings from normoxic rats and rats exposed to hypoxia for 48 hours, PE (10^{-9}-10^{-5}M) was administered in a cumulative fashion in the presence and absence of the ET_A receptor antagonist BQ123 (10^{-7}M) (n=7 in each group). In separate sets of experiments, concentration-response relationships for PE (10^{-9}M-10^{-5}M) were generated in the presence of different concentrations of ET-1 (10^{-12}M, $6X10^{-12}$ M and $3X10^{-10}$M) (n=7 in each group) or in the presence and absence of the TxA_2 receptor antagonist SQ29548 (10^{-7}M) (n=6 in each group) for endothelium-intact aortic rings from rats exposed to normoxia and rats exposed to hypoxia for 48 hours. SQ29548 was dissolved in DMSO and therefore the controls for those experiments included DMSO. Upon completion of the concentration-response protocol, the aortic

segments were removed from the hooks, dried overnight at 50°C and weighed so that tensions could be expressed as g/mg dry weight.

3.4.3 ET-1 Radioimmunoassay

ET-1 was extracted from plasma and aortic homogenates and measured according to previously published methods [28]. Immediately following decapitation, blood was collected from normoxic rats and from rats exposed to hypoxia for 12 hours and 48 hours (n=10 per condition) in 10 mL glass tubes containing EDTA (1 mg/mL) and the plasma separated by centrifugation at 1,500 × g for 15 minutes. Thoracic aortas from the same rats were excised and frozen in liquid N_2 for later extraction. Aortas from rats exposed to hypoxia for 48 hours were divided in two groups and the endothelium was removed from one half by gently rubbing the luminal surface with a wooden spatula prior to immersing both halves in liquid N_2. Aortas were homogenized for 1 minute in 2 mL of 4M guanidine isothiocyanate containing 1% trifluoracetic acid (TFA) using a polytron homogenizer. The homogenate was then centrifuged at 1,500 × g for 20 minutes at 4°C. Aliquots of the supernatant were applied to Sep-Pak C_{18} columns (Waters Associates, Milford, MA) that had been activated by washing with 100% methanol and water. The columns were then washed with water and 20% methanol. ET-1 was eluted with 90% methanol (3 × 1 mL) into a glass tube and evaporated to dryness in a Speed Vac (Savant Instruments, Farmingdale, NY). Columns to which plasma was added were washed with water and eluted with 100% methanol (3 mL) into 5mL glass tubes. Extracts or ET-1 standards were reconstituted in the radioimmunoassay buffer and incubated with rabbit anti-ET-1 serum (Peninsula Laboratories, RAS 6901) for 24 hours at 4°C, followed by a

further 24-hour incubation with 4000 cpm of ^{125}I-labeled ET-1. Bound and free radioactivity were separated by incubation with secondary anti-rabbit antibody and precipitated with 12.5% polyethylene glycol. Following centrifugation, the supernatants were removed and the radioactivity in the pellets was measured using a gamma counter. Recovery of exogenously added ET-1 was 76% by this method. Cross-reactivity of the antiserum was 5% with the related peptide ET-3, 10% with the precursor big ET-1, and none with the unrelated peptides atrial natriuretic peptide, vasopressin, and angiotensins I and II. The detection limit was 0.12 pg/tube, and the intra- and inter-assay coefficients of variation were 9 and 12% respectively.

3.4.4 Analysis of PreproET-1 mRNA

RNA extraction and cDNA cloning. The thoracic aorta from normoxic rats (n=6), rats exposed to hypoxia for 12 hours (n=6) and rats exposed to hypoxia for 48 hours (n=6) were excised and frozen in liquid nitrogen immediately following decapitation. Total RNA was extracted using TRIzol™ (Gibco BRL Life Technologies, Gaithersburg, MD) according to the method of Chomczynski and Sacchi [29].

Aortic RNA extracted from a rat in the normoxic control group was reverse transcribed and used to clone by PCR partial cDNA fragments of preproET-1 and β-actin mRNAs. One μg of total RNA was denatured by heating at 72°C for 2 min. and converted to cDNA using M-MLV Reverse Transcriptase (10 U/μL) in a reaction buffer consisting of Tris-HCl (20 mM, pH 8.4), KCl (50 mM), $MgCl_2$ (5 mM), dNTPs (1 mM), RNAase inhibitor (0.16 U/μL), $pd(N)_6$ random primers (5.0 μM).

PreproET-1 cDNA plasmid construct. A 319 bp partial cDNA fragment was generated by PCR (30 cycles) from 200 ng of total cDNA using preproET-1 specific sense (5'-CTAGGTCTAAGCGATCCTTG-3') and antisense (5'-TTCTGGTCTCTGTAGAGTTC-3') primers [30] and subcloned into the EcoR1 site of PCR II vector (Invitrogen, San Diego, Ca.). DNA sequencing indicated that this partial fragment corresponds to nucleotides 450 to 768 of the rat preproET-1 mRNA (Genbank accession number M64711) and confirmed its reverse orientation behind the T7 promoter within the vector. The full sequence of this cDNA has been submitted to Genbank (accession number AF122903).

β-actin cDNA plasmid construct. A 292 bp β-actin cDNA was also amplified by PCR (25 cycles) from 5 ng of total cDNA using β-actin specific sense primer (5'-AAGTACCCCATTGAACACGGCA-3') and antisense primer (5'-TAGATGGGCA-CAGTGTGGGTGA-3') and subcloned into the EcoR1 site of PCR II vector (Invitrogen, *San Diego, Ca.*). DNA sequencing indicated that this cDNA corresponds to nucleotides spanning exons 3 and 4 of the rat β-actin gene (Genbank accession number V01217) and confirmed its reverse orientation behind the Sp6 promoter within the vector. The full sequence of this cDNA has been submitted to Genbank (accession number AF122902).

[^{32}P]-riboprobe synthesis. The preproET-1 construct was linearized by cleavage with Styl (4 U/μg plasmid DNA, New England Biolabs, Mississauga, Ontario) downstream of the T7 promoter while the β-actin construct was linearized by cleavage with Bsu36I downstream of the Sp6 promoter. Antisense RNA for preproET-1 and β-actin were synthesized using T7 RNA polymerase (20U/μL) and Sp6 RNA polymerase (20U/μL), respectively, along with 1μg of linearized construct and 50 μCi of α-[^{32}P]-labeled CTP (400 Ci/mmol, Amersham, UK). The RNA polymerases, nucleotides and transcription

buffer were provided with the Riboprobe^{R} *In vitro* Transcription Systems purchased from Promega (Madison, WI). Plasmid DNA was digested with RNAase free DNAase (0.1U/μL) for 30 minutes at 37°C. 20 μg of yeast transfer RNA (Boerhinger-Mannheim, Laval, Quebec) was added as a carrier. The mixture was then extracted with phenol/chloroform/isoamyl alcohol and precipitated twice with ethanol. The RNA pellet was washed with 75% ethanol and resuspended in 500 μL of hybridization buffer consisting of 80% formamide, 0.4 mol/L NaCl, 1 mmol/L EDTA and 40 mmol/L PIPES (pH 6.2).

Ribonuclease protection assay. The ribonuclease (RNAase) protection assays were performed on RNA from the thoracic aortas of normoxic rats (n=7) and rats exposed to hypoxia for 12 hours (n=8) and 48 hours (n=8). RNAase protection assays for preproET-1 and β-actin were carried out simultaneously, in the same tube, on each individual aortic RNA sample from each of the rats in each group. Each reaction tube contained [^{32}P]-labeled preproET-1 (100,000 cpm) and β-actin (1000 cpm) riboprobes as well as 10 μg of total aortic RNA or yeast transfer RNA. The samples were incubated at 85°C for 5 minutes to denature the RNA and hybridization was carried out overnight at 50°C. 350 μL of RNAase digestion buffer consisting of 10 mM Tris-HCl (pH 7.5), 300 mM NaCl, 5 mM EDTA, 0.04 μg/μL RNAase A and 0.65U/μL RNAase T1 was added to each sample and incubated 1 hour at 30°C. The samples were incubated 15 minutes at 37°C in the presence of 0.13μg/μL proteinase K and 0.05% SDS. The samples were then extracted with phenol/chloroform/isoamyl alcohol, 10 μg of yeast transfer RNA was added and the mixture was precipitated with ethanol. The pellets were resuspended in loading buffer consisting of 80% (v/v) formamide, 1mM EDTA pH 8.0, 0.1% bromophenol blue and

0.1% Xylene Cyanol and 1X TBE, denatured for 3 minutes at 85°C and run on a 6% polyacrylamide 7M urea gel. The gel was then dried under vacuum and exposed to a PhosphorImager screen (FUJI, BAS-III) overnight. Protected preproET-1 and β-actin mRNA were quantified by densitometry in each rat aortic RNA sample and the level of preproET-1 mRNA in each sample was expressed as a percentage of the β-actin mRNA level within the same sample.

3.4.5 Thromboxane B_2 Radioimmunoassay

The level of the stable metabolite thromboxane B_2 (TxB_2) was measured in thoracic aorta by radioimmunoassay as an indirect measure of TxA_2 production. Thoracic aorta from normoxic rats (n=8) and rats exposed to hypoxia for 48 hours (n=8) were excised immediately after decapitation, cross-sectioned into eight segments of 2mm in length and randomly allocated to one of two groups. In one of the groups, the endothelium was removed by abrasion from all four aortic rings while the rings in the other group were left intact. The aortic rings in each group were again equally separated into two groups and pre-calibrated for 1 hour in 4 mL of Krebs solution pH 7.4 at 37°C followed by a 30 minute incubation in either the presence or absence of phenylephrine (10^{-5}M). The rings were then removed, blotted dry and frozen in liquid nitrogen. The excess serosa was removed and the rings were weighed and homogenized in 3mL of ice-cold extraction buffer containing 5mM Tris, 1.11mM ASA and 1mM EDTA. The samples were centrifuged at 1500g for 10 minutes at 4°C and the supernatants carefully transferred to a clean tube. The supernatants were rehomogenized, centrifuged at 30 000g for 45 minutes at 4°C and diluted to 8.5mL with extraction buffer. The supernatants were applied slowly to Sep-Pak C_{18} columns (Waters Associates, Milford, MA) activated by

washing with 100% methanol and water. The columns were then washed with 15% ethanol and petroleum ether. Prostanoids were eluted with 10mL of methyl formate at 4°C and dried under vacuum overnight at room temperature. Samples were washed in 1mL of methyl formate and dried overnight under vacuum. The latter step was repeated one more time. The tissue samples and TxB_2 standards (Cayman Chemicals) were resuspended in 500µL of radioimmunoassay buffer pH 7.0 containing 8.5g/L NaCl, 0.2g/L KCl, 2.2g/L Na_2HPO_4, 0.24g/L KH_2PO_4, 1.0 g/L NaN_3 and 1.0 g/L bovine gamma globulin. These were then incubated with 500cpm of [^{3}H]-TXB_2 (Amersham, U.K.) and 1:10 rabbit anti-TxB_2 serum (Cedarlane) for 2 hours at room temperature. Charcoal-dextran (1:1) was then carefully added and the mixture was centrifuged at 1500g for 15 minutes at room temperature. An aliquot from the supernatant was added to scintillation cocktail and the radioactivity was measured using a gamma counter.

3.4.6 Data Analysis

Concentration-response relationships were evaluated by comparing the tension achieved during maximum constriction and the concentration of agonist that produced 50% of the maximal response (EC_{50}). Comparisons of multiple means were performed by analysis of variance (ANOVA) corrected for repeated measures when appropriate. If the ANOVA revealed significant overall differences, variations among individual means were evaluated post-hoc using the Student-Neuman-Keuls procedure. Data are expressed as mean ± SEM, for *n* number of animals, with $p < 0.05$ representing significance.

3.5 Results

3.5.1 Effect of BQ123 and ET-1 on Aortic Contractility

Figure 3.1 illustrates the effect of the ET_A receptor antagonist, BQ123 (10^{-7}M) on the PE response of aortic rings from normoxic rats and rats exposed to hypoxia for 48 hours. BQ123 did not have any effect in rings from the normoxic group (Figure 3.1A) while it significantly reduced the contractile response to PE in rings from the hypoxic group (Figure 3.1B). To test whether or not this effect of BQ123 in the hypoxic rings was endothelium-dependent, the PE response of endothelium-intact and -denuded rings in the presence and absence of BQ123 was evaluated. BQ123 reduced the response to PE in endothelium-intact rings (Figure 3.2A) while having no effect in endothelium-denuded rings from the hypoxic group (Figure 3.2B). Taken together, these results suggest that, in aortic rings from 48-hour hypoxic rats, the effect of BQ123 is mimicked by endothelial removal and is therefore dependent on the presence of the endothelium. The mean values for maximum tensions and the inverse logarithm of concentrations associated with a 50% maximal contraction (-Log EC_{50}) during stimulation with PE, in the presence and absence of BQ123 in endothelium-intact and -denuded aortic rings from normoxic rats and rats exposed to hypoxia for 48 hours are presented in Table 3.1. BQ123 did not have any effect on the -LogEC50 under the tested conditions in the different groups.

Figure 3.1

Effect of BQ123 on PE-induced Contractility in Endothelium-Intact Aortic Rings

Concentration-response relationships for phenylephrine-induced contraction in the presence and absence of BQ123 (10^{-7}M) for endothelium-intact aortic rings from rats exposed to normoxia (**A**, ANOVA $p>0.05$ in BQ123 treated vs. untreated controls) and rats exposed to hypoxia for 48 hours (**B**, ANOVA $p<0.05$ in BQ123 treated vs. untreated controls).

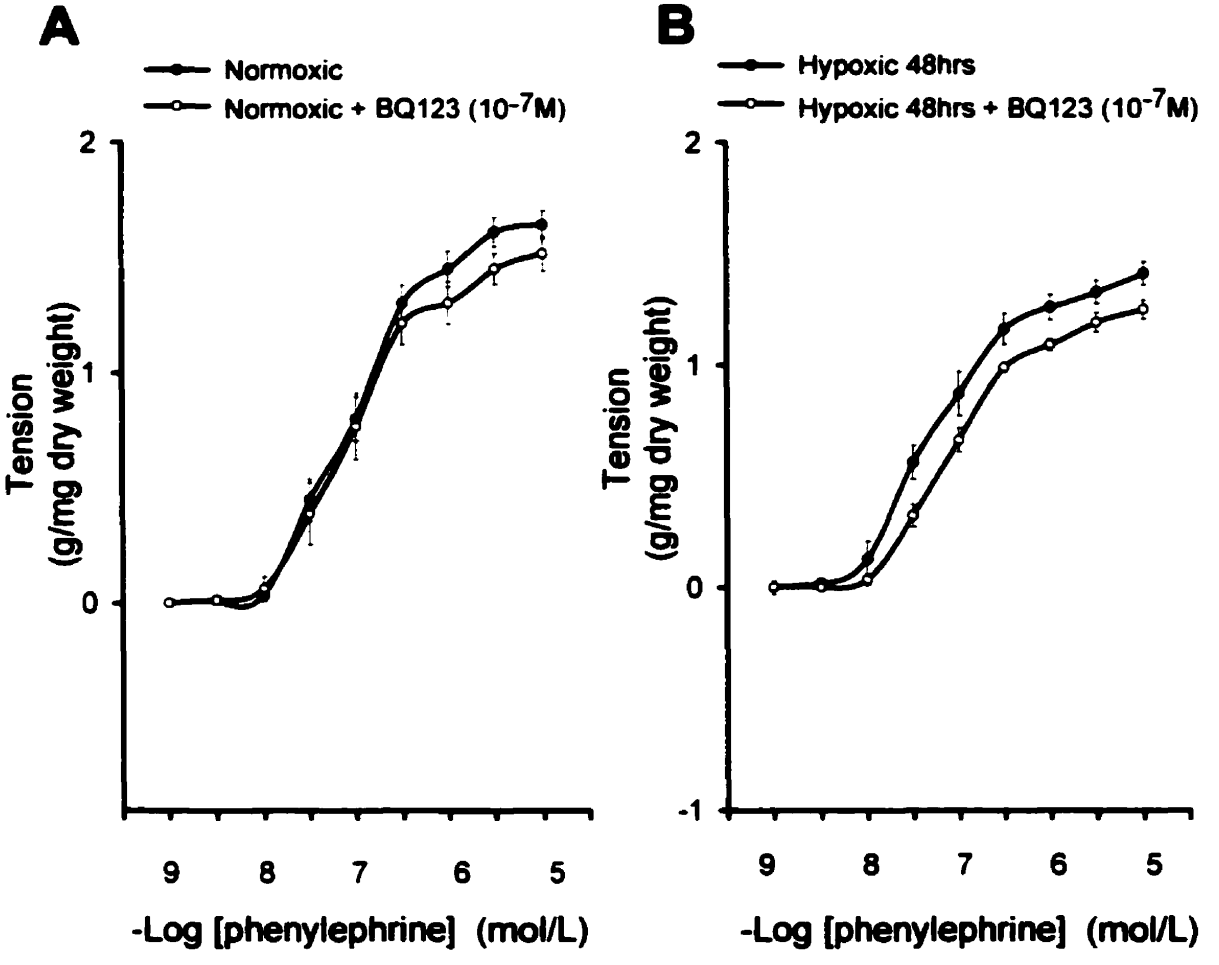

A
Normoxic
Normoxic + BQ123 (10^{-7}M)
Tension (g/mg dry weight)
2
1
0
9
8
7
6
5
-Log [phenylephrine] (mol/L)
B
Hypoxic 48hrs
Hypoxic 48hrs + BQ123 (10^{-7}M)
Tension (g/mg dry weight)
2
1
0
-1
9
8
7
6
5
-Log [phenylephrine] (mol/L)

Figure 3.2

Role of Endothelium on the Effect of BQ123 on PE-induced Contractions Following Hypoxia

Concentration-response relationships for phenylephrine-induced contraction in the presence and absence of BQ123 (10^{-7}M) for endothelium-intact (**A**, ANOVA $p<0.05$) and -denuded (**B**, ANOVA $p>0.05$) aortic rings from rats exposed to hypoxia for 48 hours.

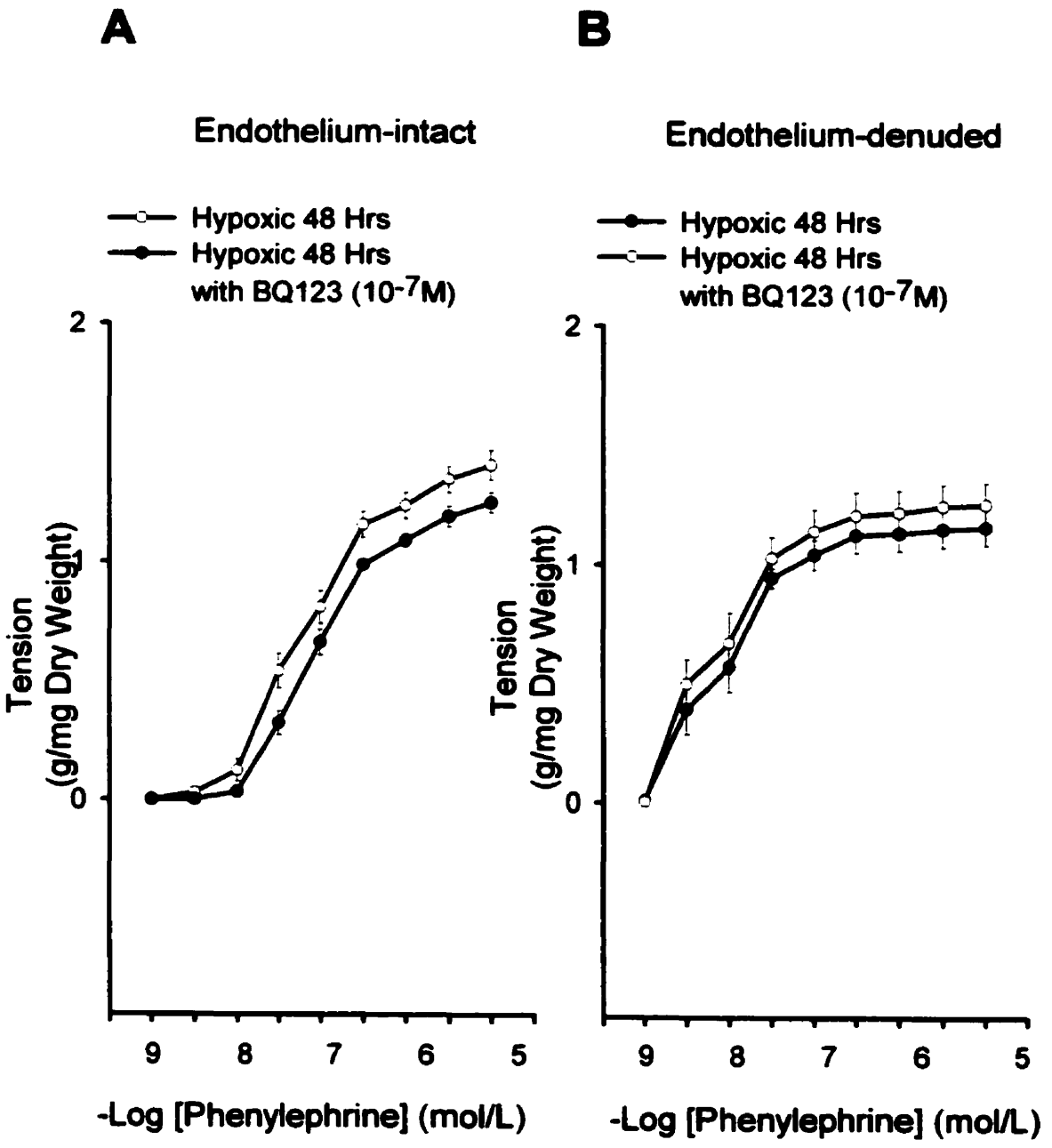

A
B
Endothelium-intact
Endothelium-denuded
Hypoxic 48 Hrs
Hypoxic 48 Hrs
with BQ123 (10-7M)
Hypoxic 48 Hrs
Hypoxic 48 Hrs
with BQ123 (10-7M)
Tension
(g/mg Dry Weight)
Tension
(g/mg Dry Weight)
2
1
0
2
1
0
9
8
7
6
5
9
8
7
6
5
-Log [Phenylephrine] (mol/L)
-Log [Phenylephrine] (mol/L)

Table 3.1

Maximum Tensions and Values of the Inverse Logarithm of PE Concentrations Associated with a 50% Maximal Contraction (-LogEC_{50}) in Aortic Rings from Normoxic Rats and from Rats Exposed to Hypoxia for 48 Hours (n=7 Animals per Group).

		Control		BQ123 (10^{-7}M)	
Condition	Endothelium	EC_{50} (-Log mol/L)	Max. Tension (g/mg)	EC_{50} (-Log mol/L)	Max. Tension (g/mg)
Normoxia	Intact	6.84 ± 0.10	1.64 ± 0.06	6.92 ± 0.16	1.56 ± 0.07
	Removed	8.02 ± 0.06$^{\Psi}$	1.89 ± 0.08$^{\Psi}$		
48 Hrs. Hypoxia	Intact	7.07 ± 0.10	1.41 ± 0.06*	6.93 ± 0.09	1.25 ± 0.04$^{\square *}$
	Removed	8.09 ± 0.12$^{\Psi}$	1.16± 0.07$^{\Psi *}$	8.10 ± 0.17$^{\Psi}$	1.22 ± 0.10

* $p < 0.05$ for difference from the corresponding value in the normoxic group.
$^{\Psi}$ $p < 0.05$ for difference from the corresponding value in endothelialized rings.
$^{\square}$ $p < 0.05$ for difference from corresponding value in control rings.

The contractile responses generated during activation with PE in endothelium-intact aortic rings from hypoxic rats and in the presence of different concentrations of ET-1 (10^{-12}M, $6X10^{-12}$M and $3X10^{-10}$M) did not display any changes in maximum tension or EC_{50}, as compared to the hypoxic control. Similar results were obtained for endothelium-intact aortic rings from normoxic rats (Table 3.2). In the current study, plasma ET-1 concentration was estimated at 10^{-12}M following 48 hours of hypoxia (see below). In other studies on hypoxia it was shown to be as high as $6x10^{-12}$M [6]. These concentrations were used *in vitro* to determine the effect of increased circulating ET-1 on aortic contractility following hypoxia. Potentiation of adrenergic responses by ET-1 at a previously reported subthreshold concentration of 3 x 10^{-10}M [31], was also tested. The mean values for maximum tension and $-LogEC_{50}$ during stimulation with phenylephrine in the presence and absence of ET-1 at these concentrations are presented in Table 3.2. The responses to phenylephrine in rings from normoxic and hypoxic rats were not altered by ET-1 at any of these concentrations, as compared to the control within each group.

3.5.2 Endothelin-1 Levels

The effect of exposure to hypoxia on plasma ET-1 concentration is illustrated in Figure 3.3A. ET-1 levels were elevated in plasma from rats exposed to hypoxia for 12 hours (2.33 pg/mL ± 0.3) and 48 hours (2.13 pg/mL ± 0.2), as compared to the levels measured in normoxic control rats (0.79 pg/mL ± 0.10; $p<0.05$ for difference). Hypoxia also increased tissue levels of ET-1 in the rat aorta (Figure 3.3B). Aortic ET-1 levels were 8.06 ± 0.94 pg/mg in normoxic rats and, 11.94 ± 1.84 pg/mg and 13.89 ± 2.03 pg/mg in rats exposed to hypoxia for 12 and 48 hours, respectively ($p<0.05$ for 48hrs

hypoxia versus normoxia). Furthermore, this increase in ET-1 levels was localized to the endothelial cell layer since endothelial ablation eliminated the difference in tissue ET-1 concentration between aortic segments from normoxic control rats and those from rats exposed to hypoxia for 48 hours.

Table 3.2

Maximum Tensions and Values of the Inverse Logarithm of PE Concentrations Associated with a 50% Maximal Contraction (-LogEC_{50}) in Aortic Rings from Normoxic Rats and from Rats Exposed to Hypoxia for 48 Hours (n=6 Animals per Group).

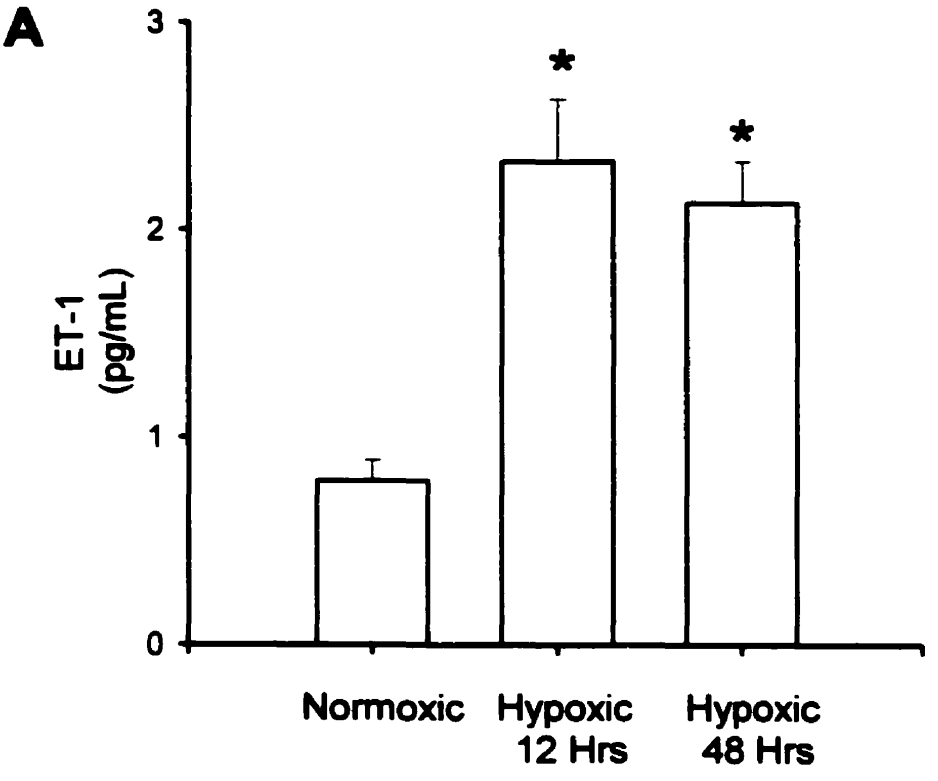
	Normoxic		Hypoxic 48 hrs	
ET-1 (mol/L)	EC_{50} (-Log mol/L)	Max. Tension (g/mg)	EC_{50} (-Log mol/L)	Max. Tension (g/mg)
Control	6.94 ± 0.17	1.62 ± 0.09	7.12 ± 0.14	1.40 ± 0.09*
10^{-12}	6.97 ± 0.17	1.59 ± 0.08	7.20 ± 0.10	1.29 ± 0.08*
$6X10^{-12}$	6.93 ± 0.13	1.72 ± 0.07	7.06 ± 0.20	1.30 ± 0.06*
$3X10^{-10}$	6.96 ± 0.24	1.73 ± 0.19	7.08 ± 0.10	1.43 ± 0.08*

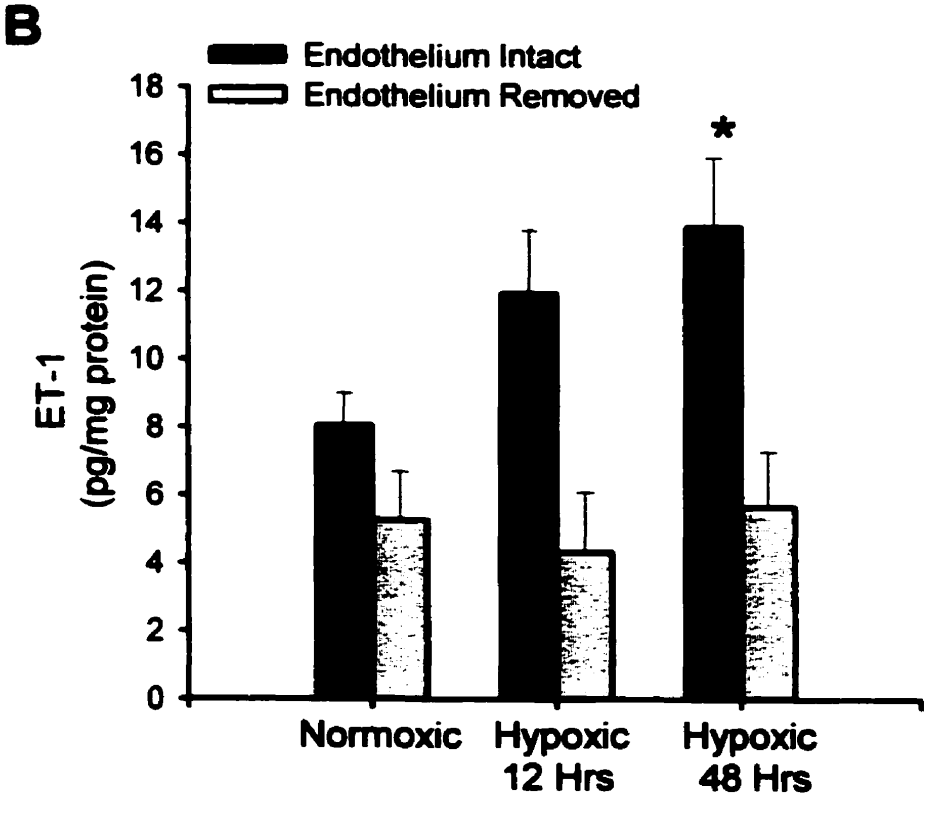
*$p < 0.05$ for difference from corresponding value in the normoxic group.
§$p < 0.05$ for difference from corresponding value in control rings.

Figure 3.3

ET-1 Levels in Rat Plasma and Aorta

A, Histogram representing circulating plasma ET-1 levels (pg/mL ± SEM) in rats exposed to normoxia (n=10), hypoxia for 12 hrs (n=10) and hypoxia for 48 hrs (n=10). t-test, * $p < 0.05$ vs. normoxic controls. **B,** Histogram representing ET-1 levels (pg/mg ± SEM) in aorta from rats exposed to normoxia (n=10), hypoxia for 12 hours (n=10) and hypoxia for 48 hours (n=10). t-test,* $p < 0.05$ vs. normoxic controls.


A
3
2
1
0
ET-1
(pg/mL)
*
*
Normoxic
Hypoxic
12 Hrs
Hypoxic
48 Hrs


B
Endothelium Intact
Endothelium Removed
18
16
14
12
10
8
6
4
2
0
ET-1
(pg/mg protein)
*
Normoxic
Hypoxic
12 Hrs
Hypoxic
48 Hrs

3.5.3 PreproET-1 mRNA expression

The results of ET-1 RNAase protection assays are illustrated in Figure 3.4A. Aortic preproET-1 mRNA levels (% of ET-1/β-actin mRNA ratio in the normoxic control) are increased in rats exposed to hypoxia for 12 hours and 48 hours ($p<0.01$ for both groups), as compared with normoxic controls shown in Figure 3.4B.

3.5.4 The effect of SQ29548 on Aortic Contractility

The effect of the TxA_2/endoperoxidase receptor antagonist, SQ29548, on the phenylephrine concentration-response relationship in endothelium intact aortic rings from normoxic and 48 hour hypoxic rats is presented in Figure 3.5A. In aortic rings from normoxic rats, SQ29548 caused a significant reduction in the contractile response to PE while it had no effect in aortic rings from rats exposed to hypoxia. The mean values ± SEM for maximum tension and EC_{50} obtained for these groups are presented in Table 3.3.

3.5.5 Thromboxane B_2 Levels

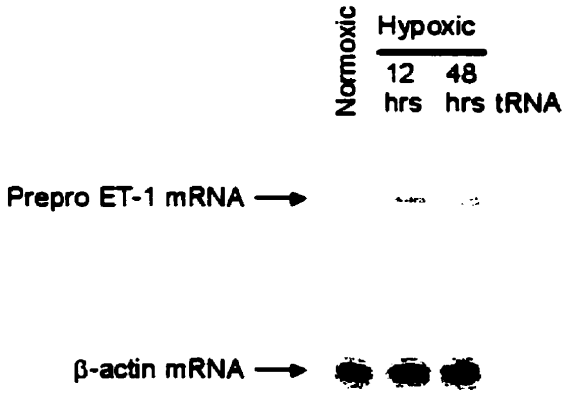
The concentration of Thromboxane B_2 (TxB_2) was measured in aortic rings from rats exposed to normoxia as well as rats exposed to 48 hour hypoxia in the presence and absence of phenylephrine (10^{-5}M). Figure 3.5B demonstrates that TxB_2 levels are not significantly changed between the normoxic and hypoxic groups. Stimulation with phenylephrine did not result in any significant variations in TxB_2 levels in normal or denuded aortic rings.

Figure 3.4

PreproET-1 mRNA Expression in Rat Aorta

A, Representative RNAase protection assay of preproET-1 mRNA carried out on total aortic RNA from rats exposed to normoxia, and hypoxia for 12 hours and 48 hrs. **B.** Histogram illustrates the % of preproET-1/β-actin mRNA ratio in the normoxic control for each group (n = 6 per group). t-test, ** $p < 0.01$ vs. normoxic controls.

A


Normoxic
Hypoxic
12 hrs
48 hrs
tRNA
Prepro ET-1 mRNA
β-actin mRNA

B

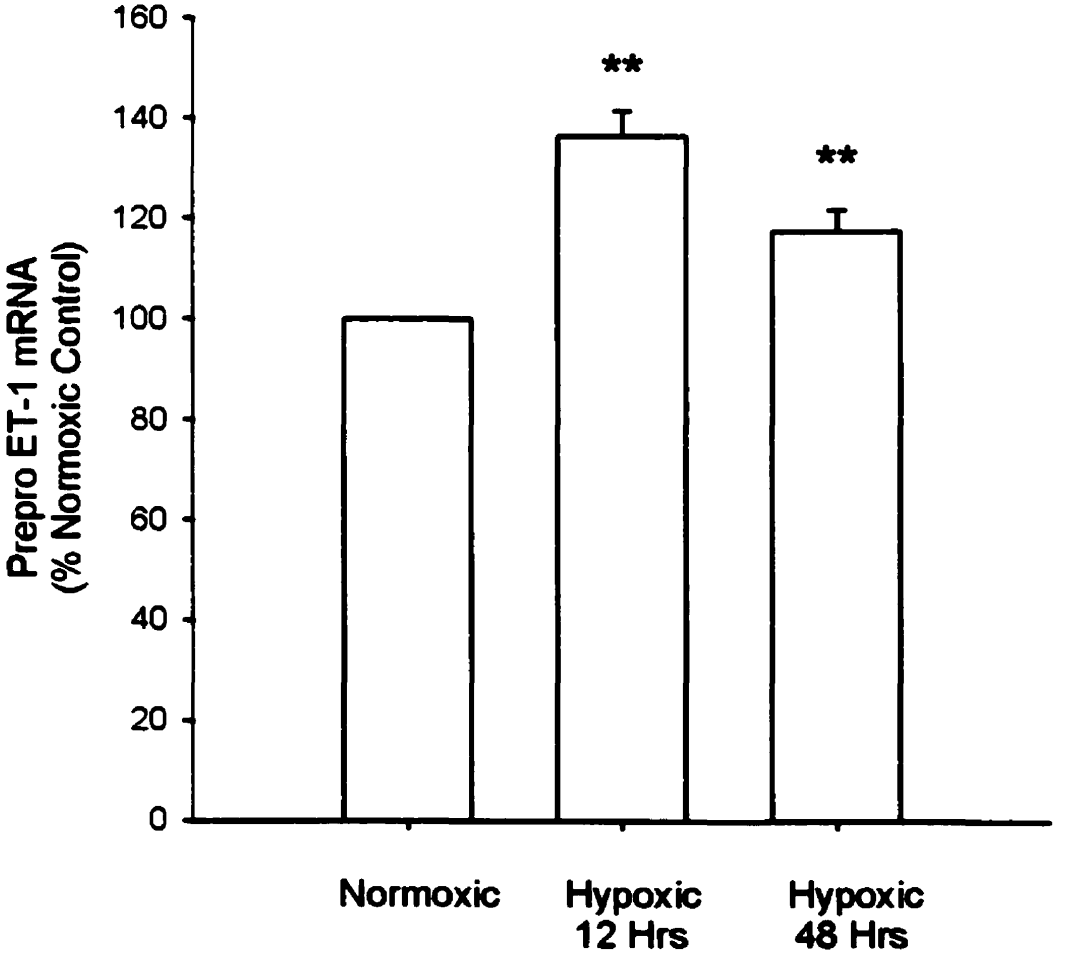

Prepro ET-1 mRNA
(% Normoxic Control)
160
140
120
100
80
60
40
20
0
**
**
Normoxic
Hypoxic
12 Hrs
Hypoxic
48 Hrs

Table 3.3

Maximum Tensions and Values of the Inverse Logarithm of PE Concentrations Associated with a 50% Maximal Contraction (-LogEC_{50}) in Aortic Rings from Normoxic Rats and from Rats Exposed to Hypoxia for 48 Hours (n=6 Animals per Group).

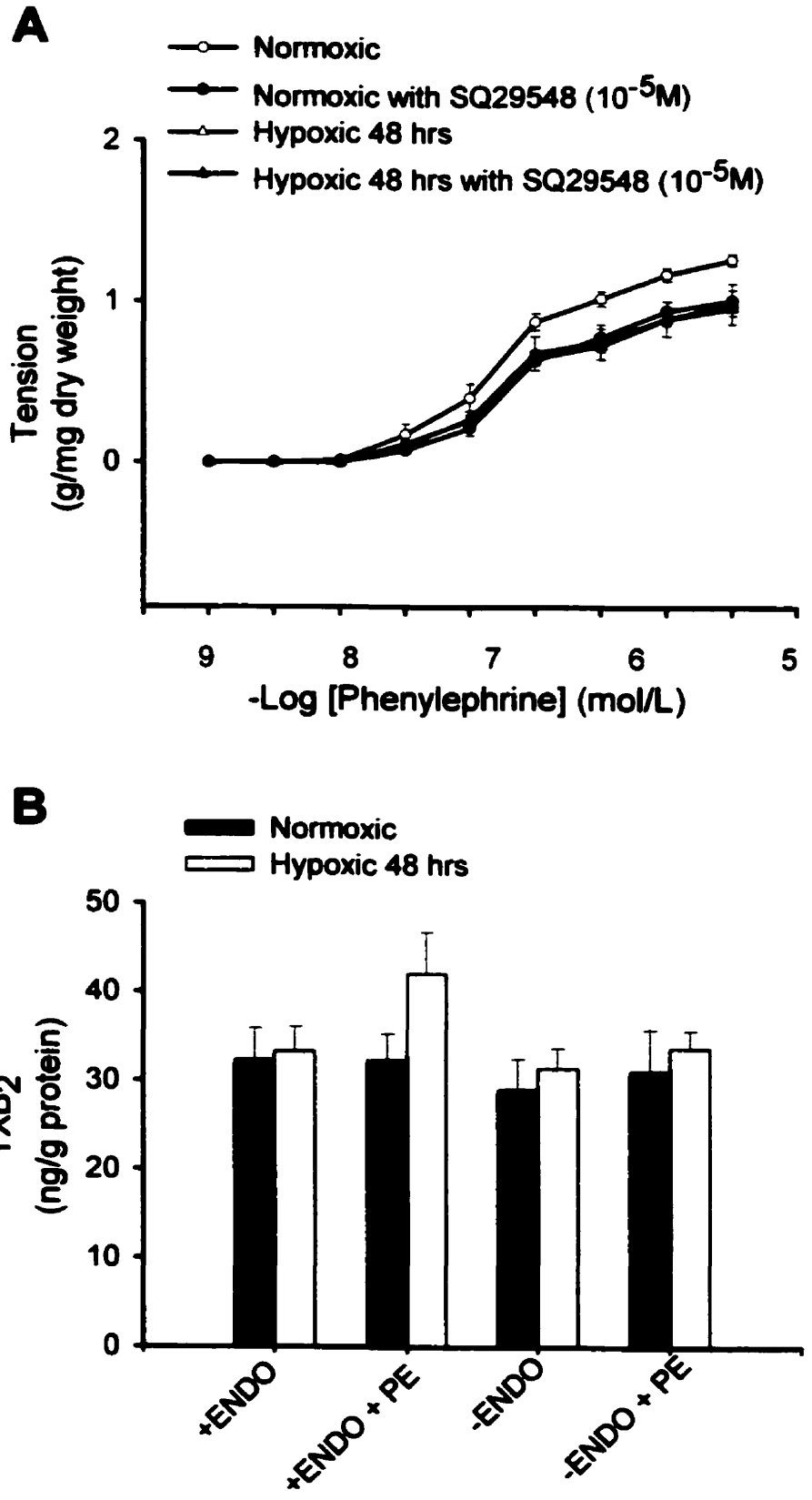
	Normoxic		Hypoxic 48 hrs	
SQ29548 (mol/L)	EC_{50} (-Log mol/L)	Max. Tension (g/mg)	EC_{50} (-Log mol/L)	Max. Tension (g/mg)
Control (DMSO)	6.60 ± 0.12	1.26 ± 0.03	6.43 ± 0.05	0.99 ± 0.13*
10^{-7}	6.53 ± 0.04	1.01 ± 0.07§	6.50 ± 0.06	0.96 ± 0.05

*$p < 0.05$ for difference from corresponding value in the normoxic group.
§$p < 0.05$ for difference from corresponding value in control rings.

Figure 3.5

The Role and Expression of TxA_2 in Rat Aorta

A, Concentration-response relationships for phenylephrine-induced contraction in the presence and absence of SQ29548 (10^{-7}M) for endothelium-intact aortic rings from normoxic rats and rats exposed to hypoxia for 48 hours (ANOVA $p<0.05$ normoxic vs normoxic +SQ29548). **B,** Histogram representing TxB_2 levels (ng/g ± SEM) in endothelium-intact and endothelium-denuded aortas from rats exposed to normoxia (n=8), hypoxia for 12 hours (n=8) and hypoxia for 48 hours (n=8) following incubation with or without phenylephrine (10^{-5}M).


A
Normoxic
Normoxic with SQ29548 (10^{-5}M)
Hypoxic 48 hrs
Hypoxic 48 hrs with SQ29548 (10^{-5}M)
Tension (g/mg dry weight)
2
1
0
9
8
7
6
5
-Log [Phenylephrine] (mol/L)
B
Normoxic
Hypoxic 48 hrs
TXB_2 (ng/g protein)
50
40
30
20
10
0
+ENDO
+ENDO + PE
-ENDO
-ENDO + PE

3.6 Discussion

The main findings of this study are that in rats exposed to hypoxia: 1) aortic contraction in response to α_1-adrenoceptor stimulation is impaired and endothelial ablation further reduces the maximum tension that can be generated; 2) the ET_A receptor-specific antagonist, BQ123, mimics the effect of removing the endothelium on maximum tension generated by aortic rings during activation by phenylephrine; 3) ET-1 concentration is increased in plasma and aorta compared to normoxic controls with the increase in aortic ET-1 being localized to the endothelial cell layer; 4) ET-1, at concentrations detected in plasma from hypoxic rats or at previously reported subthreshold levels, does not potentiate contraction to PE; 5) aortic preproET-1 mRNA levels are increased compared to those in aortas from normoxic rats; 6) aortic TxA_2 levels are unchanged compared to the normoxic controls; and 7) endothelium-dependent enhancement of α_1-adrenoceptor responses is mediated by aortic ET-1 and is not dependent on TxA_2.

Considerable indirect evidence has been previously presented which supports a role for increased ET-1 expression in the systemic vascular responses to hypoxia. Hypoxic incubation increases ET-1 levels in endothelial cells isolated from human umbilical vein [3,32], and calf coronary arteries [33]. Similarly, flow-stimulated release of ET-1 by endothelial cells from the thoracic aorta of chronically hypoxic rats was found to be greater than that by cells from normoxic rats [21]. ET-1 has been implicated, by pharmacological studies [23], as a mediator of hypoxic vasoconstriction in rat aortic segments *in vitro* and hypoxia has been shown to increase ET-1 release in isolated perfused rat mesenteric arteries [24].

When taken together, the results of these studies are compelling, however, caution is necessary in extrapolating these findings to the *in vivo* expression of native ET-1. Production of ET-1 by the vascular endothelium is subject to modulation by mechanical and neurohumoral factors [1,21,22,34] *in vivo* that are not accurately simulated in cell culture experiments or in isolated arterial segments *in vitro*. More importantly, previous attempts to demonstrate that local production of ET-1 by the systemic vasculature is increased and plays a physiological role in the circulatory adaptations to hypoxia *in vivo* have been unsuccessful. Blauw et al. [35] have reported that hypoxia (arterial oxygen saturation = 80%) increased ET-1 levels in the arterial blood but not in the forearm venous effluent of human volunteers and that infusion of ET-1 in concentrations similar to those recorded in the arterial blood did not alter forearm vascular resistance. These authors, therefore, concluded that hypoxia is a stimulus for ET-1 release from the pulmonary but not the systemic circulation and that the resulting increase in arterial plasma ET-1 concentration does not influence peripheral vascular tone. Similar conclusions regarding the source of circulating ET-1 were reached by Elton et al. [5] and Li et al. [8] who used cDNA hybridization techniques (Northern and slot blotting) to demonstrate increased steady state preproET-1 mRNA levels in pulmonary but not systemic arterial vessels (including aorta) of rats exposed to hypoxia for 48 hours. Recently, Aversa et al. [2] have reported that exposure to hypoxia for 24 hours potently induces expression of a luciferase construct containing part of the 5' flanking region of the human preproET-1 gene in the lungs of transgenic mice but produces only a small and statistically insignificant increase in aortic expression.

The inability to confirm an effect of hypoxia on ET-1 expression in the systemic circulation in these previous *in vivo* studies may be attributable to the methods used to

evaluate it. Abluminal release of endothelium-derived ET-1, or processing of Big ET-1 to the active peptide in the interstitial space [26] may result in high local concentrations, which would not be reflected in measurements of venous plasma levels. Furthermore, ET-1 is known to potentiate the response to α-adrenoceptor agonists in concentrations lower than those required to independently elicit vasoconstriction [36,37]. The absence of a change in baseline vascular resistance during arterial infusions of ET-1, therefore, does not address the possibility that locally produced ET-1 may play an important role in enhancing the response to changes in sympathetic tone. Since ET-1 is a highly potent vasoconstrictor, small changes in transcription rate, beneath the threshold for detection by Northern analysis, could result in physiologically relevant increases in protein synthesis. Finally, the reported failure of hypoxia to significantly increase ET-1 promoter activity [2] in the aorta may not be fully representative of the mechanisms which are physiologically active in this tissue because the reporter construct may lack crucial *cis*-acting regulatory elements.

Due to conflicting results and the limitations that affect interpretation of studies supporting both sides of the controversy, the question of whether or not ET-1 expression is increased in the systemic vasculature by physiologically relevant levels of hypoxia and whether its local production may contribute to the vascular responses which defend vital organ function remain unanswered. The current study was, therefore, undertaken to address this deficiency. The results confirm previous demonstrations of increased plasma ET-1 levels following hypoxia [2,4-8] and provide the first direct evidence that survivable levels of hypoxia *in vivo* induce expression of ET-1 protein and mRNA in the systemic vasculature. Smooth muscle cells in culture may be stimulated to produce ET-1 [38], and this raises the prospect that the increase in aortic ET-1 following exposure to hypoxia

may be due to its synthesis by the vascular smooth muscle. Our observation that endothelial ablation eliminated the difference in ET-1 levels between aortic rings from normoxic rats and rats exposed to hypoxia for 48 hours and the lack of an effect of BQ123 on contractility in endothelium-denuded aortic rings, however, localize the increase in ET-1 to the endothelium and exclude this possibility.

Although preproET-1 mRNA was increased in the aorta of hypoxic rats in the current study, the magnitude of the increase is small compared to that reported to occur in the lung during exposure to comparable levels of hypoxia [5]. The mechanisms by which hypoxia activates preproET-1 expression and the factors regulating its differential expression among tissues are unknown. Consensus sequences for activator protein (AP)-1 [39,40], and a 7-bp-long motif similar to the core binding site for the hypoxia inducible factor (HIF)-1 which regulates expression of erythropoietin [41] and vascular endothelial growth factor [42], have been identified in the 5' flanking sequence of the human preproET-1 gene [2]. A physiological role for these nuclear factors is supported by the finding that a 2.359 kb fragment of the 5' flanking region containing their binding sequences provides a functional promoter for the hypoxic expression of luciferase *in vivo* [2]. Further studies are needed to ascertain whether differences among tissues in the potency of hypoxia as a stimulus for the production of ET-1 [5,8] depends on additional cis-acting regulatory elements upstream of the known consensus sites are involved in determining tissue distribution.

The physiological role of endothelins synthesized and released locally in the systemic vasculature has been difficult to ascertain because of the complex pharmacological action of these peptides. The biologic activity of ET-1 is transduced by two receptor proteins, ET_A and ET_B, which mediate vasoconstriction [43] and

vasorelaxation [44] through endothelium-dependent and -independent mechanisms [45], respectively. Unraveling the importance of these receptor subtypes in a given response is rendered even more perplexing because ET-1 itself exerts a regulatory influence over their expression [46]. Furthermore, at subthreshold concentrations, ET-1 enhances the contractions elicited by other agonists [47].

Our current finding that, in endothelium-intact aortic rings from rats exposed to hypoxia, incubation with BQ123 inhibits the contractile response to phenylephrine supports a physiological role for local endothelin-1 release as a mechanism by which hypoxic impairment of vascular smooth muscle contractility may be partially compensated. BQ123 did not alter the sensitivity (EC_{50}) to phenylephrine in aortic rings from rats exposed to hypoxia as would be expected if the interaction between ET-1 and phenylephrine were synergistic and mediated solely at the level of the smooth muscle ET_A and α-1 adrenergic receptors. This presumably reflects the involvement of other pathways, which may be preserved to a variable degree during prolonged hypoxia [48], in the net response to these agonists.

The addition of exogenous ET-1 to aortic rings at concentrations similar to those observed in the plasmas of 48 hour hypoxic rats ($1X10^{-12}M$) and in patients with chronic hypoxia ($6 X 10^{-12}M$) [6] did not alter the contractile response to PE suggesting that increased circulating ET-1 does not participate in the regulation of vascular tone following hypoxia. The 38-amino acid precursor, Big ET-1, is normally cleaved into the active peptide ET-1 [1-21] by a specific endothelin-converting enzyme, ECE-1 [26]. Recently, it has been demonstrated that Big ET-1 can also be cleaved to an alternate and more potent peptide, ET-1 [1-32], by vascular matrix metalloprotease-2 (MMP-2) [49].

Vascular MMP-2 is a zinc-dependent protease normally involved in vascular remodeling whose expression is upregulated in endothelial cells in response to vascular endothelial growth factor (VEGF), a mediator stimulated by hypoxia [50,51]. This suggests that during hypoxia, both ECE-1 and MMP-2 would compete for BigET-1 in the vasculature. ECE-1 transcription and activity, however, have been shown to be inhibited by ET-1 [52] and O_2^- [53], respectively. In the current study, we have demonstrated that hypoxia increases the local production of aortic ET-1 and others have demonstrated increases in both VEGF [50,51] and O_2^- [54-59] during hypoxia. Taken together, these conditions will likely favor the processing of BigET-1 by MMP-2 as opposed to ECE-1 and thereby cause a local increase in the ratio of ET-1 [1-32] / ET-1 [1-21]. This possible scenario provides a possible explanation as to why locally produced ET-1 rather than circulating plasma ET-1 influenced contractions to PE in aortic rings from rats exposed to hypoxia.

In the normal rat aorta, ET-1 increases TxA_2 release [27,60] and antagonists to the TxA_2/PGH_2 receptor have been shown to inhibit contractions to ET-1 [27]. Normally, this effect is endothelium-independent suggesting that the increase in TxA_2 is smooth muscle derived [27]. However, in eNOS-deficient models, such as spontaneously hypertensive rats (SHRs) [61,62], animals with estrogen deficiency [63] and aortic coarctation [64], endothelium-derived TxA_2 has been shown to contribute to ET-1 and PE-induced contractions. Nitric oxide has been shown to inhibit TxA_2 synthetase activity and since we have previously reported that eNOS is downregulated following hypoxia, it is likely that endothelium-derived TxA_2 is increased in response to the observed rise in aortic ET-1 following hypoxia and contributes to ET-1-mediated endothelial enhancement of contraction. Our results, however, were unable to demonstrate a change in TxA2 levels following hypoxia.

Moreover, SQ29548 decreased the maximum tension in response to PE in aortic rings from normoxic rats, but did not have any effect in rings from hypoxic rats. In the following chapter we will present direct evidence for the induction of a NOS in vascular smooth muscle following exposure to hypoxia. It is possible that NO derived from smooth muscle following hypoxia inhibits the expected rise in TxA2 in response to ET-1 and also interferes with the effect of TxA2 on the smooth muscle.

A central role for ET-1 in mediating the adaptive responses to systemic hypoxia is emerging. In the lung, ET-1 release contributes to hypoxic vasoconstriction [65,66], the primary mechanism by which the relationship between pulmonary ventilation and perfusion is optimized. ET-1 also modulates peripheral chemoreceptor sensitivity [18,67], and thereby regulates the increase in ventilatory drive required to minimize the impact of reduced inspired oxygen concentration or parenchymal lung disease on arterial oxygen content. Through activation of vascular endothelial growth factor synthesis [68], endothelin is involved in the initiation of angiogenesis and the establishment of new capillary channels needed to maximize oxygen extraction during prolonged reductions in oxygen availability. The results of the current study suggest an additional role. The loss of reactivity to α-adrenoceptor stimulation during exposure to hypoxia will limit the available compensatory responses of the systemic vasculature. The local release of endothelin is an important mechanism by which these responses may be preserved.

3.7 References

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Chapter 4

Smooth Muscle Specific Induction of Neuronal Nitric Oxide Synthase Impairs Aortic Contractility Following Hypoxia *In Vivo*

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4.1 Foreword

We have previously demonstrated impaired contractility in both resistance [1] and conductance [2] vessels following prolonged exposure to hypoxia *in vivo*. This abnormality is endothelium-independent and is likely due to a change in gene expression in vascular smooth muscle since it persists for at least 12 hours following the restoration of normoxia [2]. It follows that this alteration is most likely manifested by vasoregulatory genes whose products are contractile proteins and/or proteins that interfere with normal contraction. The induction of nitric oxide synthase (NOS) expression and concomitant release of the potent dilator, nitric oxide (NO), in vascular smooth muscle has been previously shown to impair contractility in various disease states [3,4]. Smooth muscle cells do not normally express either the inducible nitric oxide synthase (iNOS) or neuronal nitric oxide synthase (nNOS), but are capable of doing so under certain conditions [5-9]. Moreover, the expression of both iNOS [10-12] and nNOS [13-18] have been shown to be hypoxia-inducible in certain tissues. The purpose of the following study was to test the hypothesis that following exposure to hypoxia *in vivo*, aortic smooth muscle cells express either the iNOS and/or nNOS and that NO produced by these enzymes inhibits contraction.

4.2 Abstract

The loss in vascular contractility following hypoxia is endothelium-independent and likely involves alterations in the expression of vasoregulatory genes in smooth muscle cells. The purpose of this study was to test the hypothesis that the inducible (iNOS) and/or neuronal (nNOS) nitric oxide synthases are upregulated in the aorta of rats exposed to hypoxia and that their activities consequently impair vascular contractility. Rats in the experimental group breathed a hypoxic gas mixture (FiO_2=0.1) for 12 or 48hours while the control rats were exposed to room air. The thoracic aortas were used for pharmacological, histochemical and biochemical analysis of iNOS and nNOS. Isometric tensions of endothelium-intact and -denuded aortic rings in response to cumulative doses of phenylephrine (PE, 10^{-9}-10^{-5}M) in the presence and absence of N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-5}M) were measured. L-NAME normalized isometric tension in endothelium-intact aortic rings from rats exposed to hypoxia. In endothelium-denuded aortic rings, while L-NAME enhanced the contractile response to PE in rings from the hypoxic group, it did not have any effect on aortic rings from normoxic controls. The absence of Ca^{2+}-independent NOS activity (NOS Assay), iNOS protein (Western blot) and iNOS mRNA (RT-PCR) in the aortas from the normoxic and hypoxic groups indicated that iNOS is not involved in the vascular responses to hypoxia. Despite our previous demonstration that the endothelial nitric oxide synthase (eNOS) is decreased in the aorta of rats exposed to hypoxia, Ca^{2+}-dependent NOS activity was unchanged following exposure to hypoxia, which suggested the upregulation of nNOS. This was confirmed by Western analysis, which revealed an increase in nNOS protein following 12 and 48 hours of hypoxia and this expression was localized to aortic smooth muscle by

immunohistochemistry. Neuronal NOS mRNA was also shown to be upregulated and localized to the smooth muscle layer by *in-situ* hybridization. To test whether hypoxia has a direct effect on nNOS induction, cultured vascular smooth muscle cells (SMCs) were incubated in 1% O_2. Neuronal NOS mRNA (RT-PCR) and protein (Western blot) were identified in hypoxic but not normoxic cells. The expression of nNOS was also increased in cultured human aortic SMCs following exposure to hypoxia. Based on the recently published 5'untranslated regions (UTRs) of rat nNOS (exons 1a, 1b, 1c), we found that a specific transcript containing exon 1b is induced in rat vascular SMCs during hypoxia. This was confirmed by 5'RACE conducted on aortic RNA from normoxic rats and rats exposed to 48 hours of hypoxia. Cloning and analysis of the 5'regulatory sequence upstream of exon1b revealed two putative binding sites for hypoxia inducible factor-1 (HIF-1) and six for activating protein-1 (AP-1). These novel observations indicate that nNOS is a hypoxia-inducible gene expressed in vascular SMCs and that its expression plays an important role in hypoxia-induced vascular hypocontractility.

4.3 Introduction

Hypoxia is a common clinical problem that occurs in many congenital and acquired cardiopulmonary disorders. It evokes adaptive responses from the systemic circulation, which preserve oxygen delivery (DO_2) to vital organs [19,20] and optimize intraparenchymal blood flow distribution in order to maximize tissue oxygen extraction (ERO_2) [21-26]. These reflexes are predominantly mediated by sympathoadrenal activation of the systemic vasculature and thereby normal vascular smooth muscle reactivity to adrenergic stimulation must be maintained during hypoxia. Recently, we have demonstrated that prolonged exposure to systemic hypoxia impairs smooth muscle contractility in both resistance [1] and conductance (aorta) [2] blood vessels via an endothelium-independent mechanism. The persistence of this abnormality into the post-hypoxic period [2] suggests the induction of genes whose products directly or indirectly modulate vascular contractility.

Nitric oxide (NO) is perhaps the most important locally released modulator of vascular tone. The only source of NO in the normal vasculature is the endothelial nitric oxide synthase (eNOS) expressed in the endothelium, which is, however, downregulated in the aorta following hypoxia [27] and, consequently, does not contribute to the observed decrease in contractility. Vascular smooth muscle cells are, however, capable of expressing the inducible NOS (iNOS) in response to proinflammatory cytokines [6,8] and the expression of this Ca^{2+}-independent isoform is known to contribute to impaired vascular reactivity in certain disease states [3,4]. Recently, a binding site for the hypoxia-inducible factor-1 (HIF-1) [28-32], a nuclear protein that mediates the expression of numerous hypoxia-inducible genes, has been identified in the promoter region of the iNOS gene [33]. Although

hypoxia alone does not elicit functional iNOS expression, it strongly potentiates iNOS induction by interleukin-1 (IL-1) [10,11] and interferon-γ (IFN-γ) [12]. In fact, the release of these proinflammatory cytokines is markedly increased during systemic hypoxia [34-40], and therefore suggests the possibility that iNOS may be expressed in vascular smooth muscle following hypoxia *in vivo*.

The largest and most widely distributed NOS isoform is the Ca^{2+}-dependent neuronal NOS (nNOS). The regulation of nNOS transcription and wide tissue distribution is characterized by the use of multiple promoters [5,41-43]. This expression pattern results in mRNA species with different 5'untranslated first exons alternatively spliced into a common exon 2 containing the translation initiation codon (ATG) and thereby leaving the nNOS protein structure unaltered. There are at least three different first exons (1a, 1b and 1c) reported in the rat [42] and nine others in the human nNOS gene [41], thus suggesting the likelihood of nNOS expression in a variety of tissues given the appropriate microenvironment. Previous studies have shown an increase in nNOS mRNA in central and peripheral neurons [14-16,18] as well as skeletal muscle [17] following hypoxia. While the expression of nNOS has not been reported in the smooth muscle of normal blood vessels, its presence has been demonstrated in human SMCs grown in culture [7], in atherosclerotic plaques [5] and in common carotid arterial smooth muscle of spontaneously hypertensive rats (SHR) [9]. Given that nNOS is a hypoxia-inducible gene in some tissues and that smooth muscle cells are capable of expressing this enzyme under certain conditions, we hypothesize that nNOS may also be expressed in vascular smooth muscle following hypoxia *in vivo*.

The purpose of this study was determine if the expression of iNOS and/or nNOS is induced in the smooth muscle of the aorta following prolonged exposure to hypoxia and whether these enzymes inhibit vascular contractility in response to adrenoreceptor stimulation.

4.4 Methods

4.4.1 Exposure to Hypoxia *In Vivo*

Male Sprague-Dawley rats (200-250 g) were placed in a sealed Plexiglas chamber (30 cm x 18 cm x 15 cm). The inflow of air and nitrogen into the chamber was controlled independently and the outflow was through an underwater seal. In animals exposed to hypoxia for 12 or 48 hours, the gas inflow consisted of air at a rate of 3 L/minute and nitrogen at 3 L/minute (inspired O_2 concentration = 10%). This total flow rate of 6 L/min prevented CO_2 accumulation. Normoxic rats were exposed to air only. Gas samples were drawn periodically from the chamber for analysis (AVL Instruments, Graz Austria, model 995) to ensure that the appropriate ambient PO_2 was maintained. All rats were provided with rat chow and water *ad libitum*. The temperature within the chamber was monitored using a temperature probe (Physitemp Instruments Inc., Clifton NJ, SST1) and remained the same as the surrounding room temperature throughout the exposure period. In preliminary experiments (n=4) in which blood was sampled from a cannula in the carotid artery, the arterial PO_2 averaged 38 mm Hg (range 35-42) and the arterial PCO_2 was 32 mm Hg (range 29-34). Rats were euthanized by decapitation and the thoracic aortas from normoxic rats, rats exposed to hypoxia for 12 hours and rats exposed to hypoxia for 48 hours were either immediately used for pharmacological studies, paraffin embedded for histochemistry or frozen in liquid nitrogen for biochemical studies of nNOS and iNOS.

4.4.2 Exposure to Hypoxia *in Vitro*

Rat A-10 aortic smooth muscle cells (SMCs) were purchased from American Tissue Culture Collection (ATCC, Bethesda, MD) and grown in Dulbecco's Minimum Essential Medium (D-MEM) supplemented with fetal calf serum (10%), L-glutamine (2 mM) and penicillin-streptomycin (200 U/mL). Experiments were conducted using cells from passages 17 to 22. Human aortic smooth muscle cells (SMCs) were purchased from Clonetics (San Diego, Ca.) and grown in Smooth Muscle Basal Medium (SmBM^{TM}) supplemented with 5% fetal calf serum, Gentamycin (50 μg/mL), Amphotericin-B (50 pg/mL), human recombinant fibroblast growth factor (hFGF, 2 ng/mL), insulin (5 μg/mL) and human recombinant epidermal growth factor (hEGF, 0.5 ng/mL). Experiments were conducted using human aortic SMCs from passage 5 to 10.

Rat A-10 SMCs (n=4) and human aortic SMCs (n=6) were grown to 80% confluency in 150mm culture dishes and reconstituted with fresh media prior to each experiment. Cells were exposed for 24 hours to either hypoxia or normoxia, in a sealed plexi-glass chamber placed in a 37°C tissue culture incubator. The gas inflow consisted of nitrogen (6 L/min), carbon dioxide (0.5 L/min) and air (0.2 L/min) which were controlled independently and bubbled through sterile water in order to maintain chamber humidity and prevent media evaporation. The gas outflow was continuously sampled into an O_2 and CO_2 analyzer. These parameters resulted in a hypoxic gas mixture of 1% O_2 , 5% CO_2 and the balance N_2. Cells exposed to normoxia were incubated at 37 °C in 21% O_2 and 5% CO_2. Following each exposure, cells were washed twice with Dulbecco's phosphate buffered saline, pH 7.4, and total cellular proteins or RNA was extracted for analysis by Western blot or reverse transcriptase-polymerase chain reaction (RT-PCR), respectively (see below).

4.4.3 Aortic Contractility

The thoracic aorta from normoxic rats and rats exposed to hypoxia for 48 hours (n=7 per group) were removed immediately after decapitation, cleaned of connective tissue, and cut into segments 4 mm in length. In half of the rings from each aorta, the endothelium was gently rubbed off using a wooden spatula while in the other half the endothelium was left intact. Aortic segments were mounted on stainless steel hooks connected to Grass force/displacement transducers (model FT03) in 15 mL jacketed organ baths containing modified Krebs solution (in mM: Na^+ 143.0; K^+ 5.9; Ca^{2+} 2.5; Mg^{2+} 1.2; Cl^- 153.9; HCO_3^- 25.0; SO_4^{2-} 1.2; $H_2PO_4^-$ 1.2; dextrose 10.0) and tension was gradually adjusted to 2g over 1 hour. The solution in the bath was kept at 37°C and bubbled with a 95% O_2, 5% CO_2 gas mixture. In all of the rings, the response to the endothelium-dependent vasodilator, acetylcholine (Ach, 10^{-4} M), was assessed following contraction with phenylephrine (PE, 10^{-5}M) to ascertain the integrity of the endothelium. The absence of vasorelaxation was taken as evidence of endothelial damage or ablation. Changing the solution in the organ bath washed out PE and ACh and tension was allowed to return to the previous baseline value of 2g. Concentration-response relationships for PE (10^{-9}M-10^{-5}M) in the presence and absence of L-NAME (10^{-5}M) were then generated for both endothelium-denuded and intact aortic rings from rats exposed to normoxia and rats exposed to hypoxia for 48 hours. Upon completion of the concentration-response protocol, aortic segments were removed from the hooks, dried overnight at 50°C and weighed so that tensions could be expressed as g/mg dry weight. Tensions generated under a given condition by aortic rings from any given animal were averaged and the averaged values were treated as single observations.

4.4.4 NOS Activity Assay

The thoracic aorta from normoxic rats (n=6), rats exposed to hypoxia for 12 hours (n=6) and rats exposed to hypoxia for 48 hours (n=6), were dissected immediately following decapitation and protein homogenates were prepared in ice-cold 50 mM Tris buffered saline supplemented with EDTA (1mM) and EGTA (1mM). The samples were spun at 14 000g and the supernatants were quantified for protein by the Bradford method [44] using bovine serum albumin as a standard. The conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline was assessed in these supernatants in the presence (Ca^{2+}-dependent activity) and absence (Ca^{2-}-independent activity) of $CaCl_2$ (0.6 mM) using the NOSdetect™ Assay Kit purchased from Stratagene (La Jolla, CA). Briefly, 10 μL of the supernatants was added to a reaction mixture consisting of Tris-HCl (25mM pH 7.4), BH_4 (3μM), FAD (1μM), FMN (1μM), NADPH (1mM), [^{3}H]-L-arginine (Amersham, Arlington Heights, Illinois. 0.2 μCi/μL), calmodulin (0.1μM) and incubated for 1 hour at 37°C. The reaction was terminated with HEPES buffer (50mM, pH 5.5) containing EDTA (5mM). The mixture was then filtered through the provided Dowex resin and the radioactivity in 100μL of the eluate was measured in a liquid scintillation counter. For each sample, [^{3}H]-L-citrulline production was also assayed in the presence of the non-specific NOS inhibitor L-NAME (10^{-3}M) and this value was used as a control to subtract all non-NOS-derived activity that may be present. In all experiments, triplicate measurements for each sample were averaged and this value was treated as a single observation.

4.4.5 Western Blot Analysis

The thoracic aorta from normoxic rats (n=4), rats exposed to hypoxia for 12 hours (n=4) and rats exposed to hypoxia for 48 hours (n=4), were excised and frozen in liquid nitrogen immediately following decapitation. Aortic proteins were extracted by homogenization in a 50mM Tris-HCl buffer pH 7.4 supplemented with PMSF (100 μg/mL), aprotinin (0.5 μg/mL), leupeptin (0.5 μg/mL), pepstatin (0.5 μg/mL) and trypsin-chymotrypsin inhibitor (0.5 μg/mL). Following exposure to either normoxia (21% O_2) or hypoxia (1% O2) for 24 hours, rat A-10 SMCs and human aortic SMCs were washed twice with Dulbecco's phosphate buffered saline pH 7.4 and total cellular proteins were also extracted in the above buffer. Cells were further lyzed by gentle repetitive passes through a 25-gauge syringe. Samples obtained from tissues and cells were then briefly centrifuged at 2500 rpm to sediment cellular debris and protein concentration was quantified according to the Bradford method using bovine serum albumin as a standard. Proteins were then separated on a 4-12% SDS-polyacrylamide gradient gel at a constant voltage of 125 V and transferred onto nitrocellulose for 2 hours at a constant current of 250mA in transfer buffer (pH 9.9) containing methanol (20%), $NaHCO_3$ (3.8mM) and Na_2CO_3 (7.9mM). The nitrocellulose membranes were blocked overnight at 4°C in a solution containing Tris (20mM), NaCl (0.5M), dry no-fat milk (7% w/v), FCS (1%) and Tween-20 (0.1%) pH 7.5. Membranes containing aortic proteins were then probed for 2 hours at room temperature with either an iNOS (1:500) or nNOS (1:500) specific monoclonal antibody purchased from Transduction Laboratories (Lexington, KY) while those with proteins from rat A-10 SMCs or HASMCs were probed for nNOS (1:500). The membranes were briefly (10 min.) washed 3 times in a buffer composed of Tris (20mM), NaCl (0.5M) and Tween-20

(0.1%) pH 7.5. Incubation with the HRP-labeled goat anti-mouse secondary antibody (1:1000) was allowed for 1 hour at room temperature and the 3 wash steps were repeated. The peroxidase reaction was developed using an enhanced chemiluminescence ECL™ immunodetection kit purchased from Amersham (UK) and the membranes were exposed to Kodak resin-coated Hyperfilm. Bands for nNOS were quantified by densitometry and nNOS protein levels were expressed in arbitrary units of density/mm^2. Staining with Ponceau Red confirmed the electrotransfer efficiency and was used as an indirect control for loading.

4.4.6 Immunohistochemistry

Thoracic aorta from normoxic rats and rats exposed to 48 hours of hypoxia were fixed by immersion in 4% paraformaldehyde and embedded in paraffin. These sections were analyzed by indirect immunohistochemistry using a polyclonal antibody to nNOS protein (Transduction Laboratories, Lexington KY) and a horseradish peroxidase labeled anti-rabbit secondary antibody. Diaminobenzadene (DAB) was used as the substrate for horseradish peroxidase and tissues were counterstained with hematoxylin and eosin. *In situ* hybridization was also carried out on these tissue sections as previously described [45] using an [^{35}S]-labelled antisense riboprobe complementary to exon 2 of nNOS mRNA. [^{35}S]-labelled sense riboprobe was used as a negative control.

4.4.7 Reverse Transcriptase (RT)-PCR

Total RNA from thoracic aortas and cultured SMCs was extracted using TRIzol™ (Gibco BRL Life Technologies, Gaithersburg, MD) according to the method of

Chomczynski and Sacchi [46]. One μg of RNA from each sample was denatured by heating at 72°C for 2 min. and converted to cDNA using M-MLV reverse transcriptase (10U/L) in a reaction buffer consisting of Tris-HCl (20mM,), RNase inhibitor (0.2 U/L) and pd $(N)_6$ random primers (5.0 mM). The PCR mixture, which consisted of cDNA (1μg), primers (0.62 μM), Tris-HCl (20 mM, pH 8.4), KCl (50mM), $MgCl_2$ (1.5mM) and Taq Polymerase (0.03 U/μL) was then subjected to 40 cycles of heat denaturation (94°C, 1 min.), annealing (60°C, 1 min.), extension (72°C, 1.5min.) and a final additional extension for 10 min. at 72°C. The PCR products and appropriate markers (1μg) were run on a 1-2 % TAE agarose gel.

Detection of iNOS mRNA in aorta. The thoracic aorta from rats treated with LPS (7 mg/kg) for 12 hours (n=4), normoxic rats (n=4), rats exposed to hypoxia for 12 hours (n=4) and those from rats exposed to 48 hours (n=4) of hypoxia were excised and frozen in liquid nitrogen immediately following decapitation. The cDNA was then amplified by PCR using sense (5'GGATCCTGCCACCTTGGAGTT 3') and antisense (5'AGCCATGACCTTCCGCAT TAG 3') primers. These primers amplified a 765 base pair partial fragment spanning from the junction of exon 8 and 9 through to exon 14 of the rat iNOS mRNA (Genbank Accession D14051).

Detection of nNOS mRNA in rat A-10 SMCs. Following reverse transcription of total RNA extracted from rat A-10 SMCs exposed to either normoxia or hypoxia, the cDNA was amplified by PCR using sense (5' ACACCGAGCTCATCTATGGCG 3') and antisense (5'TGCCTGTCTCTGTGGCGTAG A3') primers. These primers amplified a 1100 base pair partial fragment spanning exons 6 to 13 of the rat nNOS mRNA (Genbank Accession X59949).

Specific 5'-untranslated region (UTR) of nNOS mRNA in rat A-10 SMCs. Total mRNA extracted from rat A-10 cells exposed to either normoxia or exposed to hypoxia for 24 hours was reverse transcribed and amplified by PCR using published sense primers specific for the recently identified alternatively spliced exon 1 sequences of rat nNOS: exon 1a (5' AGCGGGATCCACAGCCCTGGAACT3'), exon 1b (5'GACTGAGGGGCGACACTAC CATGC 3'), and exon 1c (5' CACCACAGCCTCTGGA ATGAAAGA 3'). A common antisense primer (5' GGCGT-CATCTGCTCATTCCGATTC 3') specific to exon 2 of the rat nNOS was used to amplify each alternatively spliced exon.

4.4.8 5' RACE

Following the extraction of total aortic RNA as previously described from normoxic rats (n=4) and rats exposed to hypoxia for 48 hours (n=4), the samples were treated with RNAase-free DNAase I (2.5 μg/μL) for 15 min. at 37°C to remove genomic DNA contaminants. The mixture was extracted with phenol-chloroform and precipitated with ethanol. Total RNA was resuspended in DEPC-treated sterile double distilled water and quantified by spectrophotometry. The 5'/3' RACE kit was purchased from Boehringer Mannheim (Laval, Quebec) and used for the rapid amplification of the nNOS 5' cDNA ends. Briefly, 2 μg of RNA from the thoracic aorta of rats exposed to normoxia or 48 hour hypoxia was converted to cDNA using an nNOS (Genbank accession X59949) specific antisense primer (AS1, 0.62μM 5' GGCGTCATCTGCTCA TTCCGATTC 3') and AMV reverse transcriptase (1 U/μL) in a reaction buffer consisting of Tris-HCl (50mM, pH 8.5), $MgCl_2$ (8 mM), KCl (30mM), DTT (1 mM) and dNTPs (1mM). This mixture was

incubated at 55°C for 60 min. and the reaction was terminated by an additional incubation at 65°C for 10 min. The cDNA was purified using *High Pure* filter tubes and polyadenylated with terminale transferase (0.4 U/μL) and dATP (0.08mM) in a reaction mixture consisting of Tris-HCl (10mM, pH 8.3), $MgCl_2$ (1.5 mM) and KCl (50mM) incubated at 37 °C for 20 min. The tailed cDNA was PCR amplified at 55°C annealing temperature using an upstream nNOS specific antisense primer (AS2, 0.62 μM 5'CCTTGGGTGGCATGATTTCAACGTG 3'), the provided oligo dT-anchor primer and Expand™ High Fidelity polymerase (0.62 U/μL) purchased from Boehringer Mannheim (Laval, Quebec). The amplified cDNA was diluted (1:20) and 1μL was reamplified by PCR at 65°C annealing temperature using another upstream nNOS specific antisense primer (AS3, 0.62 μM 5'GTCAAGGTTGACCAGGCAGACGTCA 3'), the provided PCR anchor primer and Expand™ High Fidelity polymerase (0.62 U/μL). The PCR products were analyzed on a 1% ethidium bromide stained agarose gel. Distinct bands were excised, subcloned into the pCR II vector (Invitrogen, San Diego, CA) and sequenced.

4.4.9 Cloning of Rat nNOS Promoter

Using two antisense primers adjacent to one another (primer 1= 5'TGCGAAGCCGTCCCTTGGCATGGTA3' and primer 2= 5'CATCTGGAGAGAC TGCAGCTCTAATC3') specific for exon1b and the GenomeWalker™ kit purchased from Clontech Laboratories (Palo Alto, CA), various lengths of the upstream 5' regulatory sequences were generated by PCR, subcloned into the pCR II vector (Invitrogen, San Diego, CA) and sequenced. The GenomeWalker™ kit consisted of 5 rat genomic DNA libraries, each digested with a specific restriction endonuclease namely EcoRV, ScaI, DraI,

PvuII and SspI. The prior ligation of a special adapter to the ends of these genomic DNA fragments by the manufacturer allowed the amplification of unknown sequences upstream of exon1b through a series of nested PCRs. The products were cloned into the pCRII vector and sequenced. Sequence analysis for the presence of putative binding sites for HIF-1 and AP-1 was conducted using DNAMan software (Lynon Biosoft).

4.5 Results

4.5.1 Aortic Contractility

In Figure 4.1, the concentration-response relationships for PE in the presence and absence of L-NAME in endothelium-intact and -denuded aortic rings from normoxic rats and rats exposed to hypoxia for 48 hours are illustrated. As expected, L-NAME enhanced the tension and sensitivity of endothelium-intact aortic rings from normoxic rats during activation with PE (Figure 4.1A). Despite our previous demonstration that eNOS is downregulated following hypoxia [27], treatment with L-NAME also significantly ameliorated contractility to PE in endothelium-intact aortic rings from hypoxic rats (Figure 4.1B). In fact, the concentration-response relationship for PE in the presence of L-NAME in endothelium-intact rings in the hypoxic group (max tension=1.79 ± 0.08 g/mg,; -$LogEC_{50}$=7.34 ± 0.06) was not significantly different ($p>0.05$, ANOVA) from that obtained for the normoxic group (max tension=1.93 ± 0.13 g/mg; -$LogEC_{50}$=7.44 ± 0.05). In endothelium-denuded rings, while the treatment with L-NAME did not elicit any change in the normoxic group (Figure 4.1C), it significantly enhanced the response to phenylephrine in the hypoxic group (Figure 4.1D). In endothelium-denuded rings from hypoxic rats, the maximum tension and -$LogEC_{50}$ during activation with PE was 1.49 ± 0.12 g/mg in L-NAME treated versus 1.27 ± 0.09 g/mg in untreated rings and 8.54 ± 0.05 in L-NAME treated versus 7.99 ± 0.04 in untreated rings in the hypoxic group, respectively. The mean ± SEM values for -$LogEC_{50}$ and maximum tension during phenylephrine-induced contraction for all of the contractility experiments are presented in Table 4.1.

Figure 4.1

Aortic Contractility

Concentration-response relationships for phenylephrine-induced contraction in the presence and absence of L-NAME (10^{-5}M) for endothelium-intact (**A** and **B**, $p<0.05$ ANOVA, respectively) and-denuded (**C** and **D**, $p>0.05$ and $p<0.05$ ANOVA, respectively) aortic rings from rats exposed to normoxia and rats exposed to hypoxia for 48 hours.

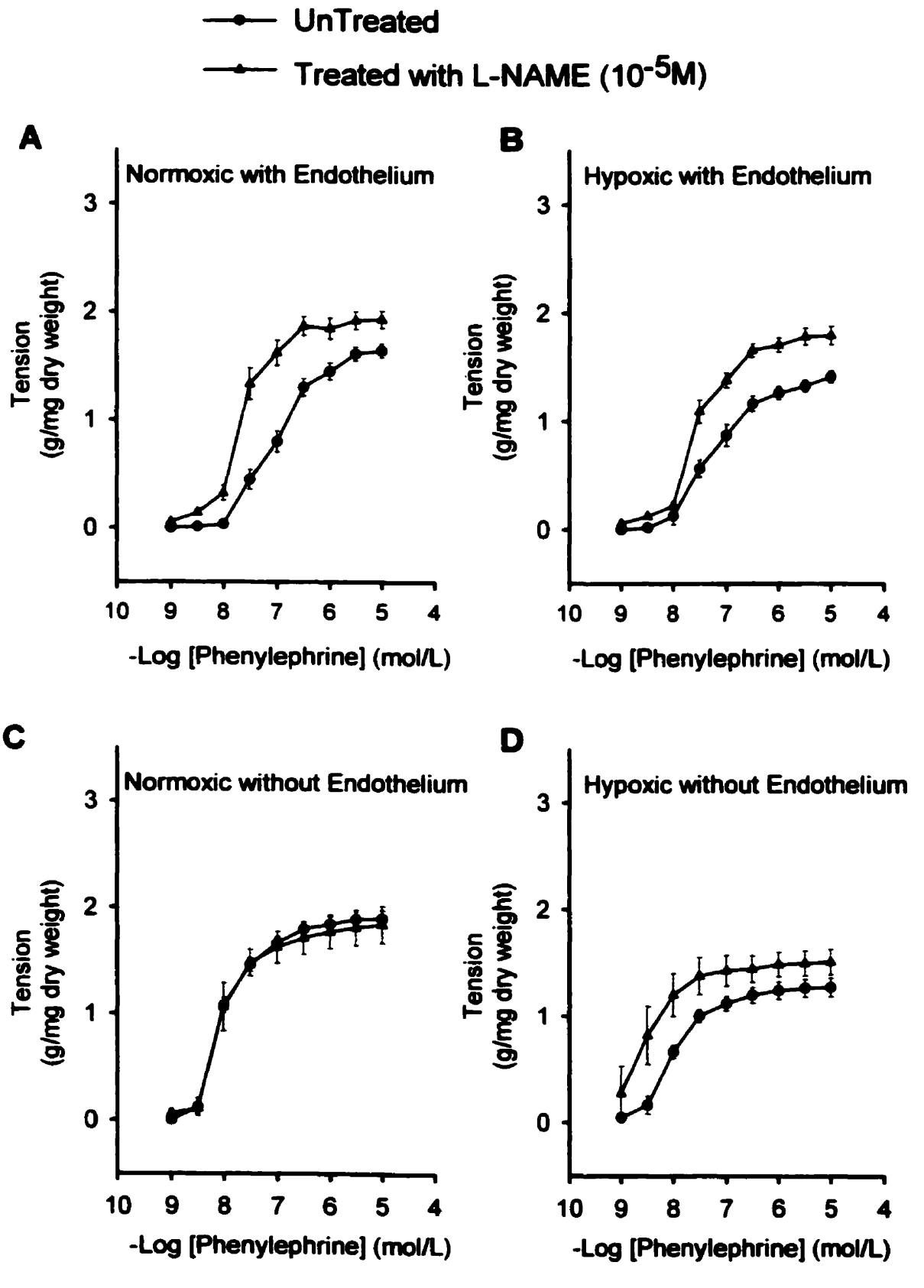

UnTreated
Treated with L-NAME (10^{-5}M)
A
Normoxic with Endothelium
B
Hypoxic with Endothelium
C
Normoxic without Endothelium
D
Hypoxic without Endothelium
Tension (g/mg dry weight)
-Log [Phenylephrine] (mol/L)
0
1
2
3
10
9
8
7
6
5
4

Table 4.1

Maximum Tensions and Values of the Inverse Logarithm of PE Concentrations Associated with a 50% Maximal Contraction (-logEC_{50}) in Aortic Rings from Normoxic Rats and from Rats Exposed to Hypoxia for 48 hours (n=7 Animals per Group).

		Control		L-NAME (10^{-5}M)	
Condition	Endothelium	EC_{50} (-Log mol/L)	Max. Tension (g/mg)	EC_{50} (-Log mol/L)	Max. Tension (g/mg)
Normoxia	Intact	6.84 ± 0.10	1.64 ± 0.06	$7.44 \pm 0.05^{\S}$	$1.93 \pm 0.13^{\S}$
	Removed	$8.02 \pm 0.06^{\Psi}$	$1.89 \pm 0.08^{\Psi}$	$8.03 \pm 0.05^{\Psi}$	1.85 ± 0.17
48 Hrs. Hypoxia	Intact	7.07 ± 0.10	$1.41 \pm 0.06^{*}$	$7.34 \pm 0.06^{\S}$	$1.79 \pm 0.08^{\S}$
	Removed	$7.99 \pm 0.04^{\Psi}$	$1.27 \pm 0.09^{*\Psi}$	$8.54 \pm 0.05^{*\Psi\S}$	$1.49 \pm 0.12^{*\Psi\S}$

* $p < 0.05$ for difference from the corresponding value in the normoxic group.
$^{\Psi}$ $p < 0.05$ for difference from the corresponding value in endothelialized rings.
§ $p < 0.05$ for difference from corresponding value in control rings.

4.5.2 NOS Activity

Despite our previous demonstration that the Ca^{2+}-dependent eNOS is downregulated following hypoxia [27], Ca^{2+}-dependent NOS activity remained unchanged ($P>0.05$, ANOVA) in the aorta of rats exposed to hypoxia for 12 hours (20.29 ± 2.43 pmol/min/mg) and rats exposed to hypoxia for 48 hours (20.95 ± 2.67 pmol/min/mg) as compared to the normoxic controls (22.38 ± 2.74 pmol/min/mg) (Figure 4.2). Ca^{2+}-independent NOS activity was negligible in all experimental groups suggesting the absence of the iNOS isoform.

4.5.3 iNOS Protein and mRNA Expression

Figure 4.3 illustrates the results of two other key molecular techniques that were used to test for the possible induction of iNOS in the aorta following hypoxia. In Figure 4.3A, a representative western blot of iNOS (n=4) in rat aortic homogenates from rats exposed to normoxia, 12 hours hypoxia and 48 hours hypoxia is depicted. An immunoreactive band was only present in the positive control lane, which consisted of an activated macrophage cell line homogenate. The presence of iNOS mRNA was tested for by RT-PCR and our results indicate that while the aorta is capable of expressing iNOS mRNA in response to LPS, it does not express this NOS isoform following exposure to hypoxia.

Figure 4.2

NOS Activity

Histogram representing the mean ± SEM of Ca^{2+}-dependent and –independent NOS activity in aortic homogenates from rats exposed to normoxia, 12 hrs of hypoxia and 48hrs of hypoxia.

$p>0.05$, ANOVA for hypoxic groups versus normoxic control.

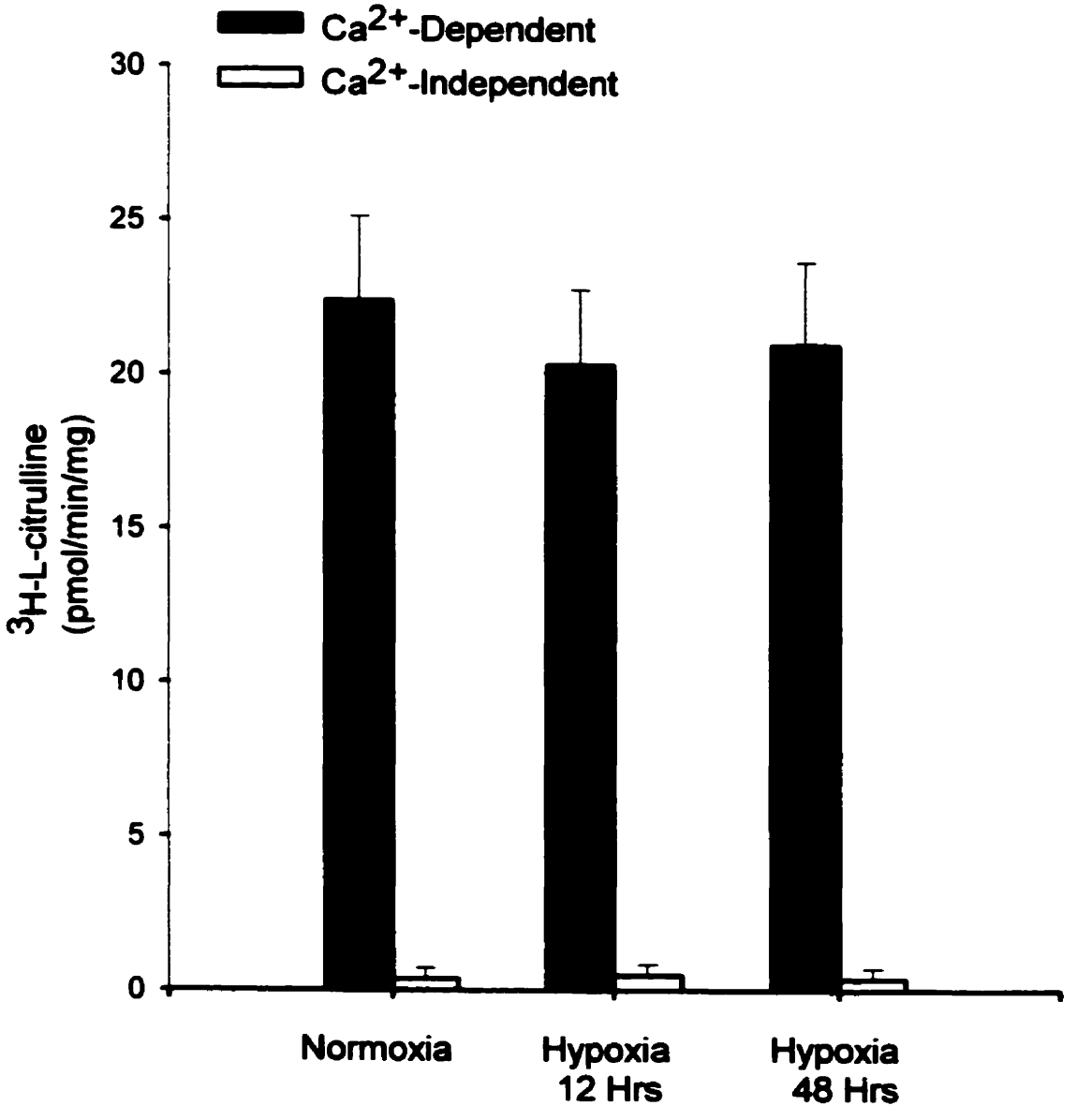
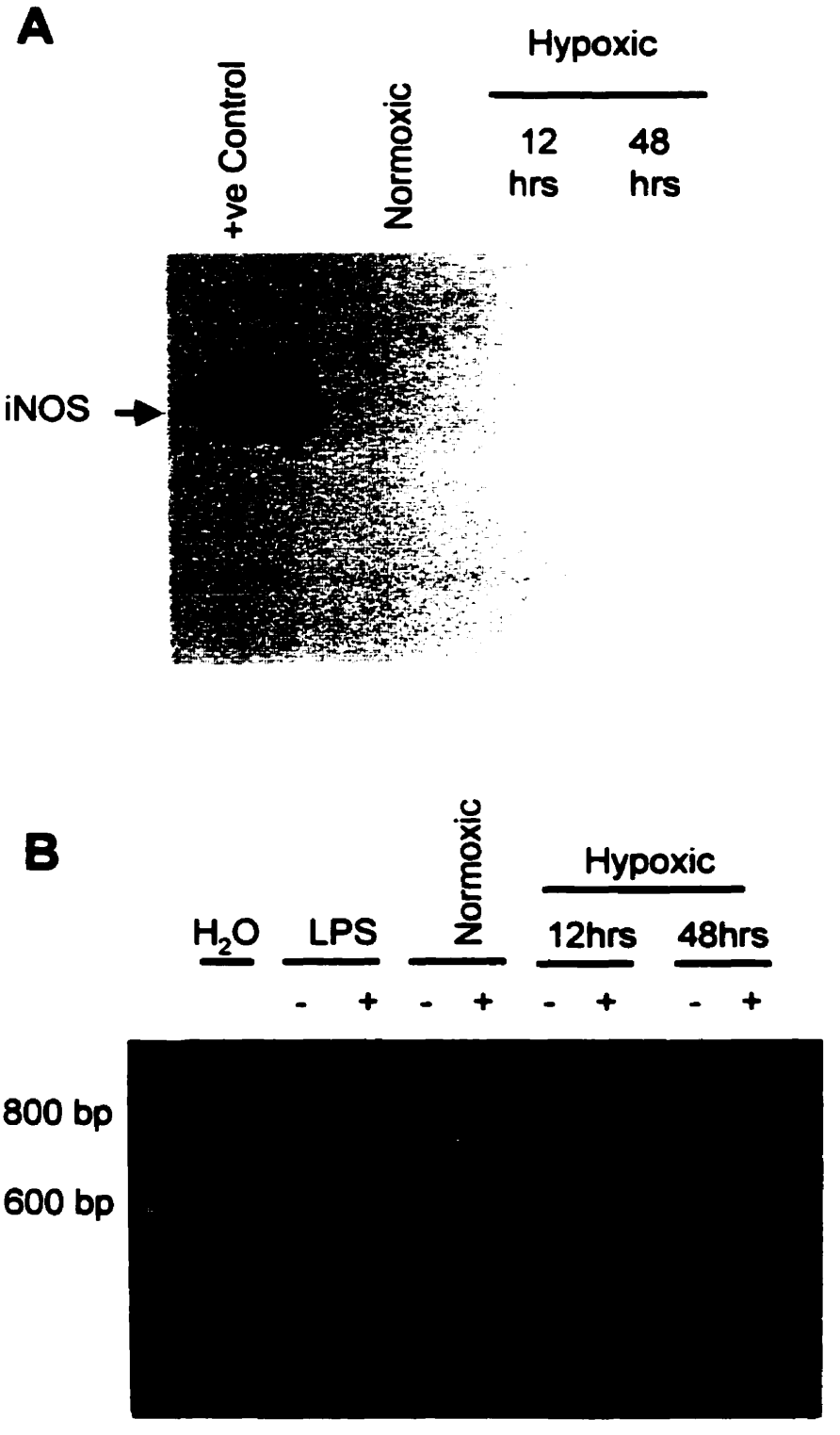

Ca2+-Dependent
Ca2+-Independent
30
25
20
15
10
5
0
3H-L-citrulline
(pmol/min/mg)
Normoxia
Hypoxia
12 Hrs
Hypoxia
48 Hrs

Figure 4.3

Aortic iNOS protein and mRNA Analysis

A, Representative western blot of the inducible nitric oxide synthase (iNOS) carried out on proteins from thoracic aortas of a normoxic rat and from rats exposed to 12 hrs and 48 hrs of hypoxia. A protein lysate of activated macrophages was used as a positive control.

B, Representative reverse transcriptase PCR of rat iNOS mRNA in aortas from rats exposed to LPS (7mg/Kg) for 12 hours, rats exposed to normoxia and hypoxia for 12hrs and 48 hrs.


A
Hypoxic
+ve Control
Normoxic
12 hrs
48 hrs
iNOS
B
Normoxic
Hypoxic
H2O
LPS
12hrs
48hrs
- + - + - + - +
800 bp
600 bp

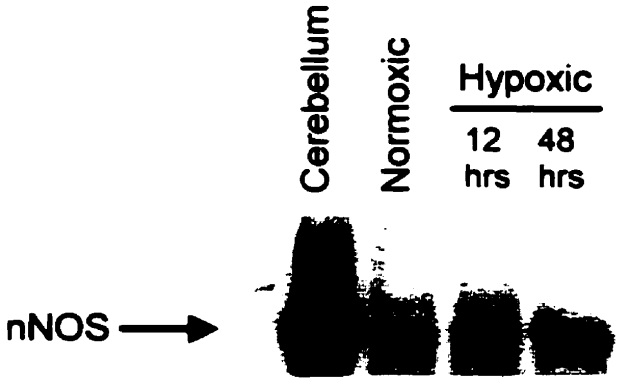
4.5.4 nNOS Protein and mRNA Expression

Aorta. As demonstrated by a representative western blot (n=4) on aortic homogenates from normoxic and hypoxic rats in Figure 4.4, nNOS was upregulated in the aorta following 12 hours of hypoxia ($p<0.05$, as compared to the normoxic group) and its level remained elevated in aorta from rats exposed to 48 hours hypoxia ($p<0.05$, as compared to the normoxic group). The difference in nNOS protein level in the aorta of rats exposed to hypoxia for 12 hours versus 48 hours was not significant ($p>0.05$). The aorta of rats exposed to 48hrs of hypoxia displayed positive staining for nNOS protein and mRNA in its medial and adventitial layers indicating that the expression of this enzyme following hypoxia is predominantly localized to the smooth muscle (Figure 4.5). The media of the aorta from normoxic controls did not show any staining, while the serosa was stained. As for the localization of nNOS mRNA by in situ hybridization, the medial and adventitial layers from both normoxic and hypoxic rats were positive for nNOS, except that qualitatively the relative amount of grains in the smooth muscle layer was greater in the aorta from rats exposed to hypoxia than that in normoxic rats. As a negative control, using [^{35}S]-labelled cRNA probe in the sense orientation did not give any signal (data not shown).

Figure 4.4

Aortic nNOS Protein Expression

Representative Western blot of neuronal nitric oxide synthase (nNOS) carried out on proteins from the rat cerebellum, thoracic aortas from normoxic rats, 12hr hypoxic rats and rats exposed to 48hrs of hypoxia. Histogram illustrates the mean ± SEM aortic nNOS protein levels (arbitrary units of optical density) for each group (n = 4 per group). **, $p < 0.01$ vs. normoxic controls.


Cerebellum
Normoxic
Hypoxic
12 hrs
48 hrs
nNOS

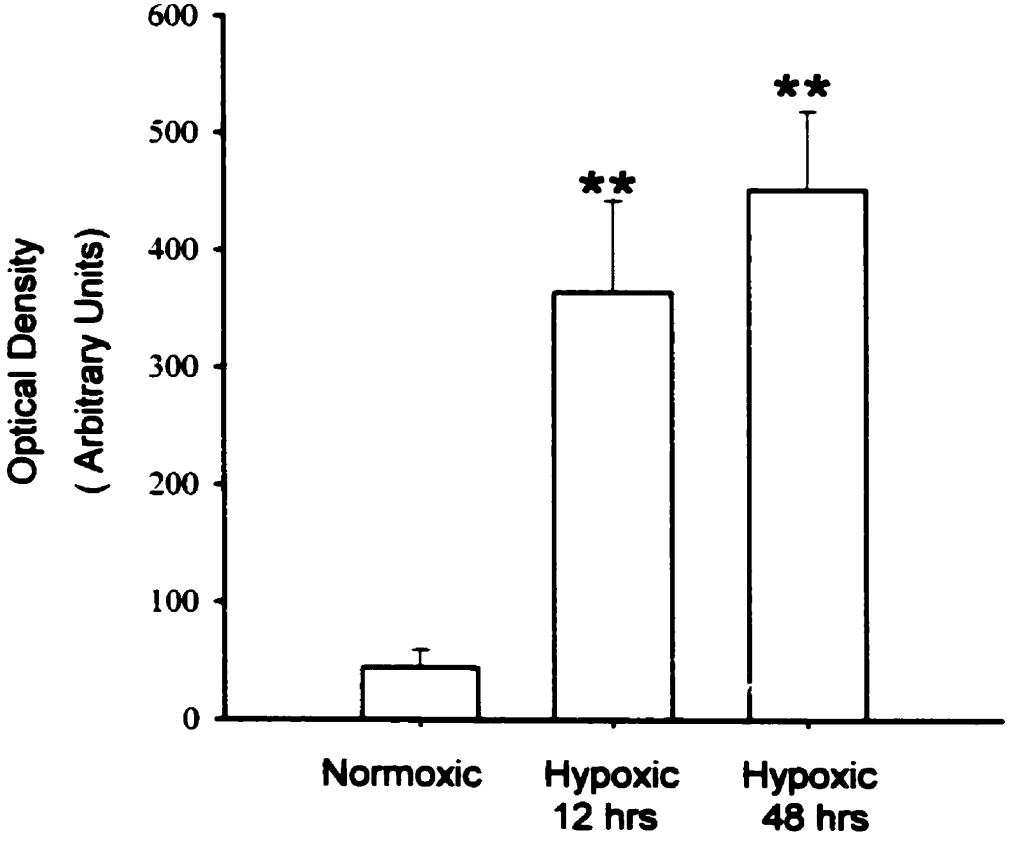
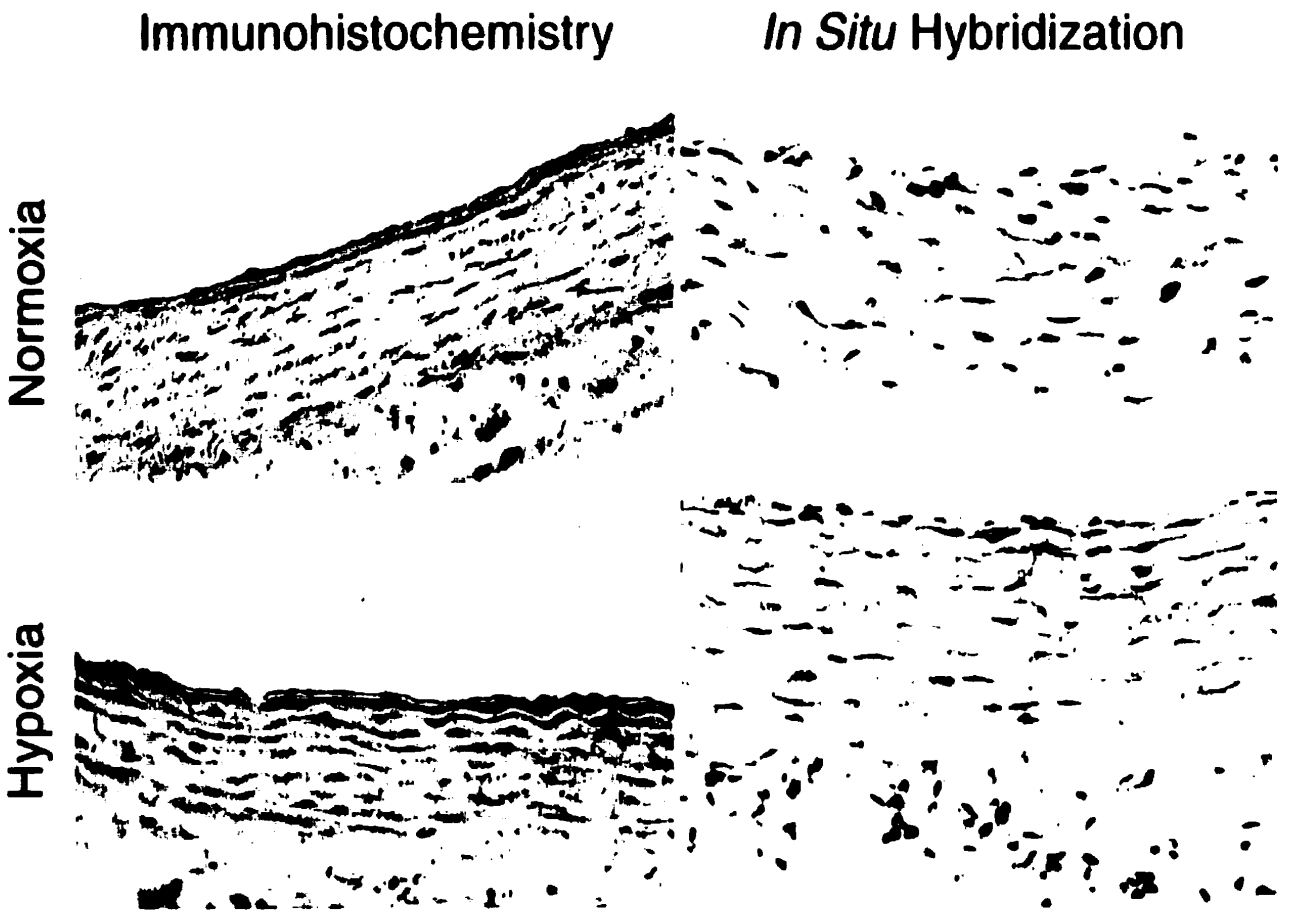

Optical Density
(Arbitrary Units)
600
500
400
300
200
100
0
**
**
Normoxic
Hypoxic
12 hrs
Hypoxic
48 hrs

Figure 4.5

Localization of aortic nNOS Protein and mRNA

Immunohistochemistry and *in situ* hybridization of nNOS protein and mRNA, respectively, on rat aortic tissue sections from normoxic rats and rats exposed to 48 hours of hypoxia.


Immunohistochemistry
In Situ Hybridization
Normoxia
Hypoxia

Aortic Smooth Muscle Cells in Culture. The nNOS protein and mRNA were induced in rat A-10 SMCs following exposure to hypoxia (Figure 4.6 A & B, respectively).These results suggest that the response of SMCs are not caused by non-specific effects due to mechanical or neurohumoral stimuli on these cells in vivo. As demonstrated in Figure 4.7, cultured human aortic SMCs were found to express nNOS under normoxic conditions, albeit at a lower level than that observed following exposure to hypoxia. Using published sense and antisense primers for the three previously identified exon 1 sequences of rat nNOS (exon 1a, 1b and 1c), we tested for their presence in rat A-10 SMCs exposed to either normoxia or hypoxia for 24 hours. Figure 4.8 illustrates the ethidium bromide gels which demonstrate that transcripts for all three of these exons are present in total RNA extracted from the cerebellum, while only exon 1b is expressed in rat A-10 aortic SMCs following exposure to hypoxia.

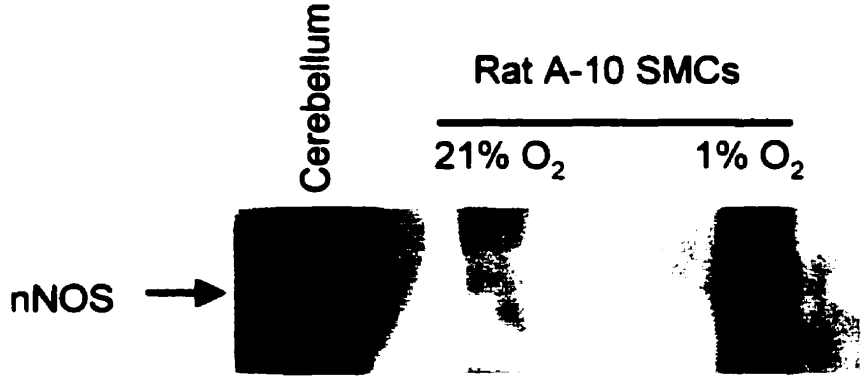
Figure 4.6

nNOS Protein and mRNA in Cultured Rat aortic A-10 SMCs

A. Representative Western blot of neuronal nitric oxide synthase (nNOS) carried out on proteins from the rat cerebellum, and A-10 aortic smooth muscle cells exposed to normoxia (21%O_2) and hypoxia (1% O_2).

B. Representative reverse transcriptase PCR of nNOS mRNA in rat cerebellum and rat A-10 aortic SMCs exposed to normoxia (21% O_2) and hypoxia (1% O_2).

A


Cerebellum
Rat A-10 SMCs
21% O_2
1% O_2
nNOS

B

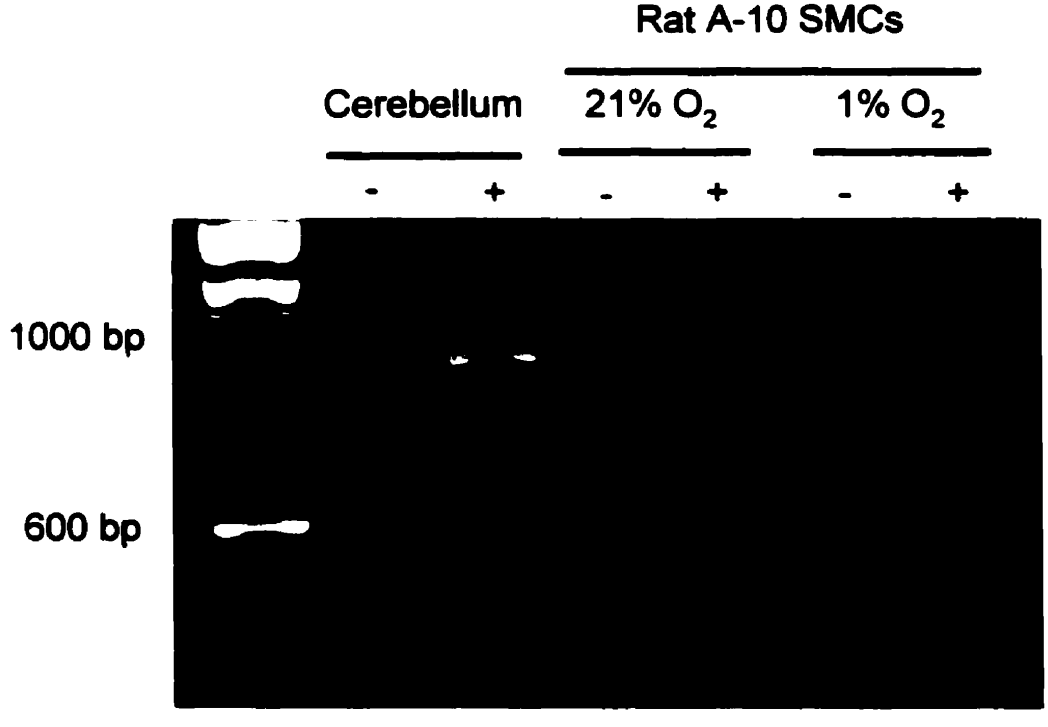

Rat A-10 SMCs
Cerebellum
21% O_2
1% O_2
- + - + - +
1000 bp
600 bp

Figure 4.7

nNOS Protein in Cultured Human Aortic SMCs

Representative western blot of nNOS protein carried out on proteins from human aortic SMCs exposed to normoxia (21% O_2) and hypoxia (1% O_2).

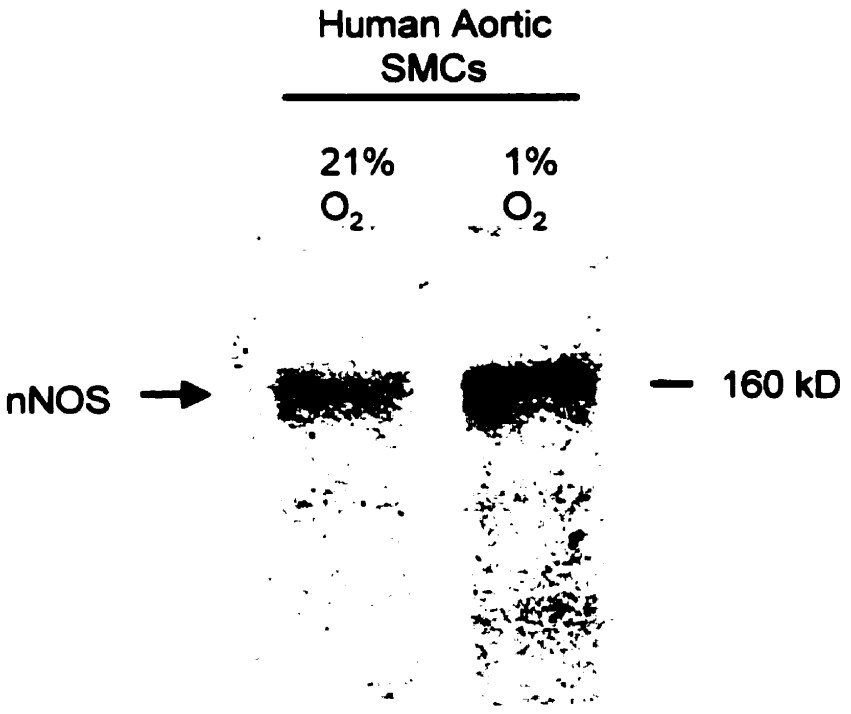
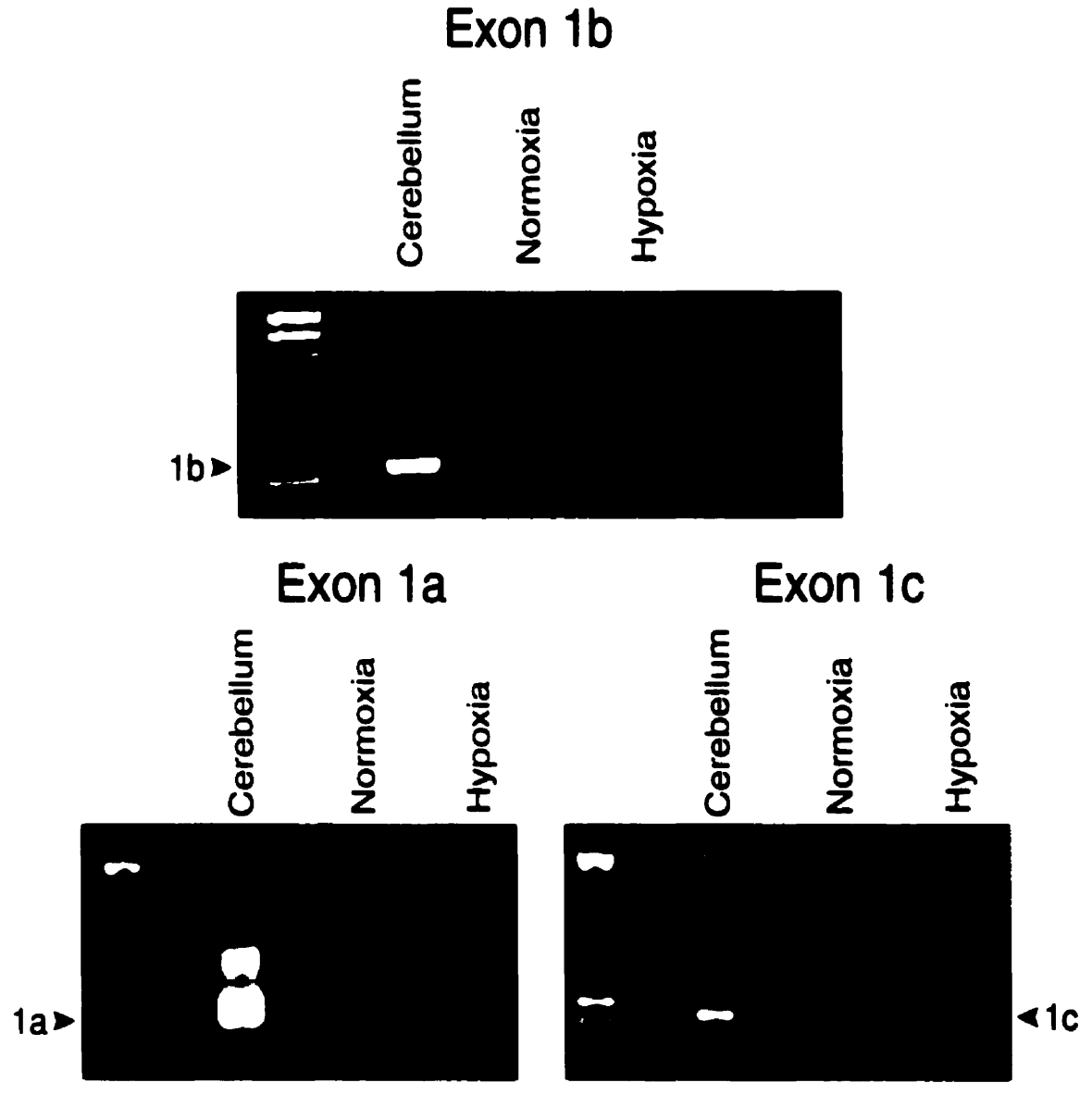

Human Aortic SMCs
21% O_2
1% O_2
nNOS
160 kD

Figure 4.8

Exon 1 Transcript of nNOS in Cultured Rat Aortic A-10 SMCs Following Hypoxia

Representative reverse transcriptase PCR of alternatively spliced exon 1 variants (1a, 1b and 1c) expressed in rat cerebellum and in rat A-10 SMCs exposed to normoxia (21% O_2) and hypoxia (1% O_2).


Exon 1b
Cerebellum
Normoxia
Hypoxia
1b
Exon 1a
Cerebellum
Normoxia
Hypoxia
1a
Exon 1c
Cerebellum
Normoxia
Hypoxia
1c

4.5.5 5' RACE

The Rapid Amplification of 5'DNA Ends was conducted in order to ascertain whether or not other previously unidentified exon 1 variants may be expressed in the aorta of rats exposed to hypoxia. Similar bands were obtained in both groups. Sequence analysis of these bands confirmed that they were identical to the previously reported exon 1b sequence of rat nNOS. (Data not shown)

4.5.6 Rat nNOS Promoter

Figure 4.9 illustrates DNA fragments of various lengths representing the 5'regulatory sequence upstream of and including exon 1b of rat nNOS that were amplified by PCR using specific antisense primers to exon 1b and rat genomic DNA libraries digested with specific restriction enzymes. Figure 4.10 illustrates the sequence determined for the 5'end region of rat nNOS spanning from exon 1b till −1283 bases upstream of the transcription initiation site. In rat smooth muscle following hypoxia, exon1c of rat nNOS is part of the 5'regulatory sequence upstream of the first transcribed exon 1b. This sequence was analyzed for putative binding sites for transcription factors most involved in hypoxic gene expression, namely HIF-1 and AP-1. Two putative response elements for HIF-1 were detected, one present on the negative (-) DNA strand (3'—5'direction) and the other on the positive (+) DNA strand (5'—3'direction) as indicated in Figure 4.10. Six putative sites for Ap-1 are also illustrated.

Figure 4.9

Cloned DNA Fragments Upstream of Exon 1b

Overlapping DNA fragments amplified by PCR of rat genomic libraries digested with EcoRV, ScaI, DraI, PvuII and SspI and representing regulatory sequences of rat nNOS upstream of exon 1b.

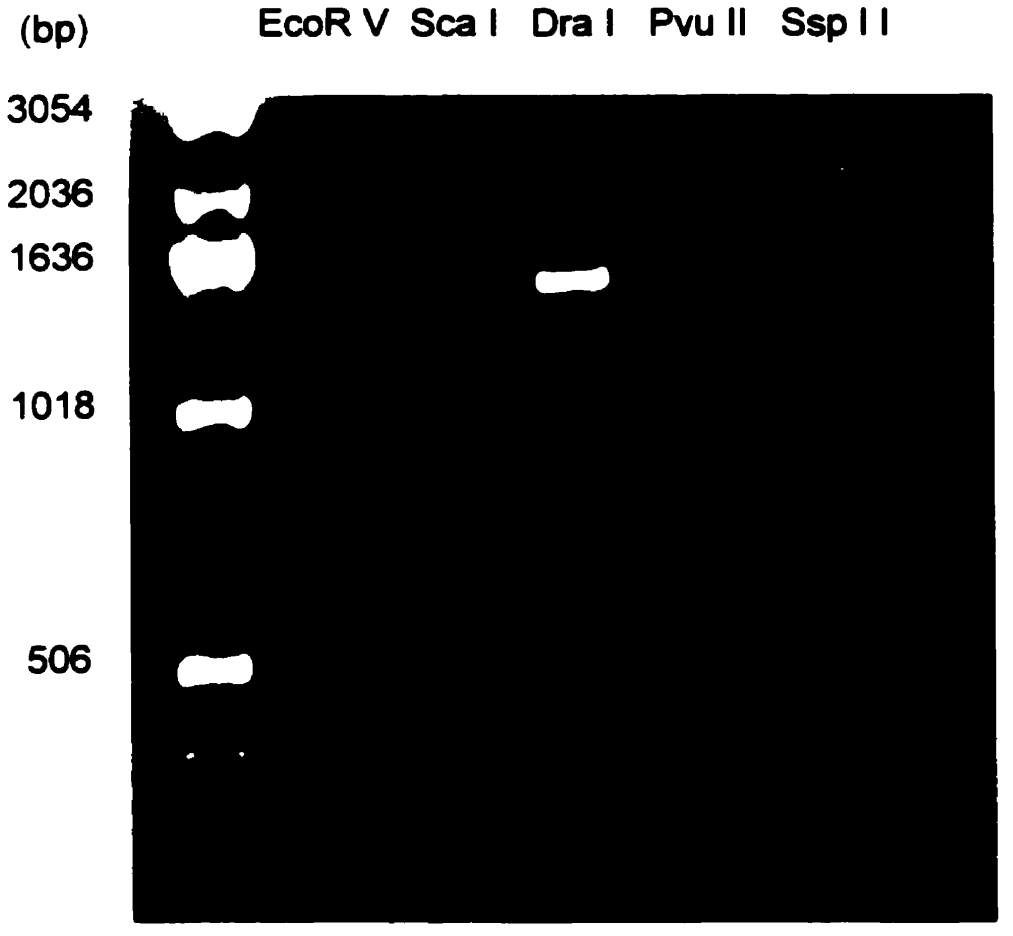
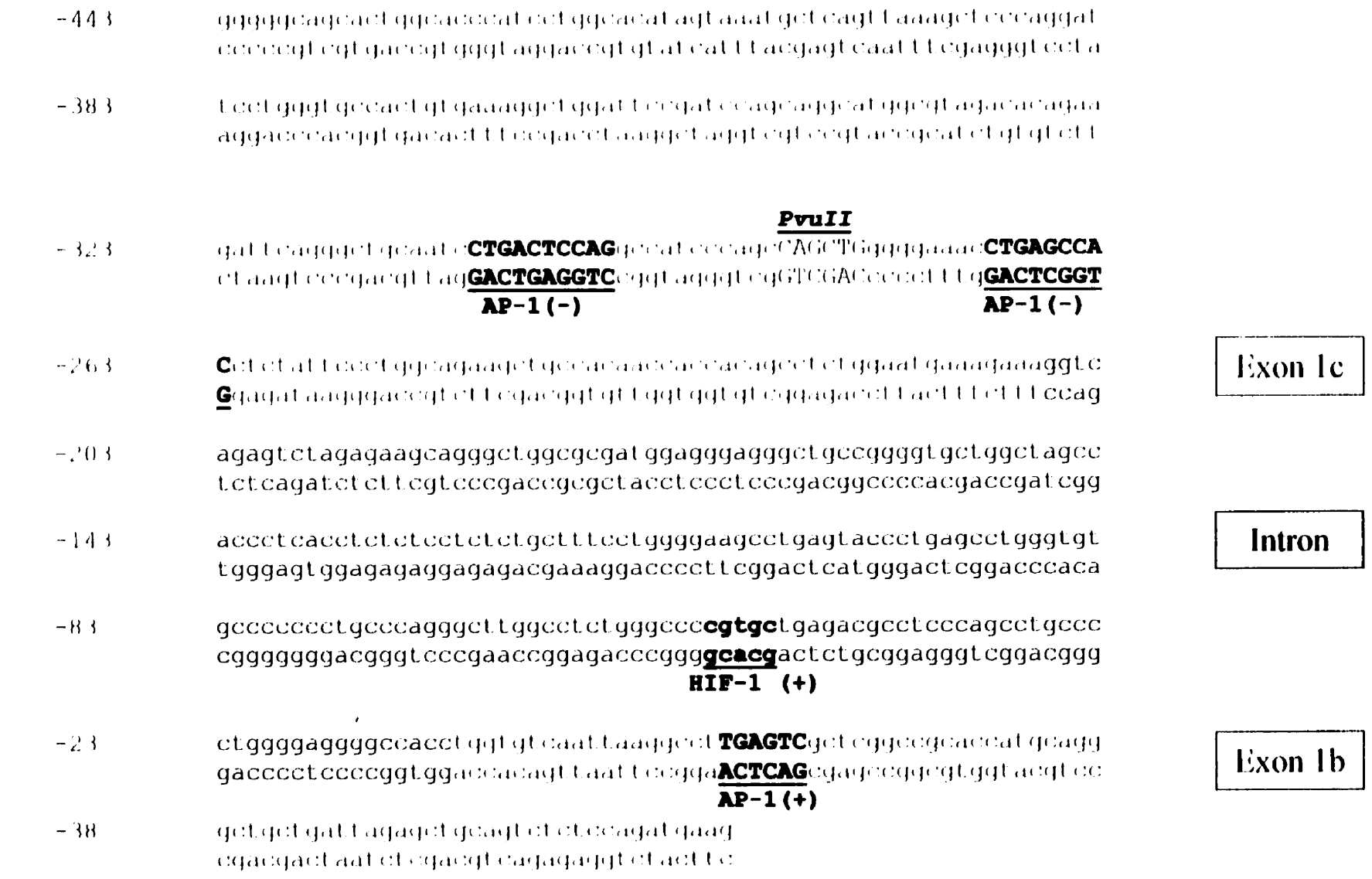
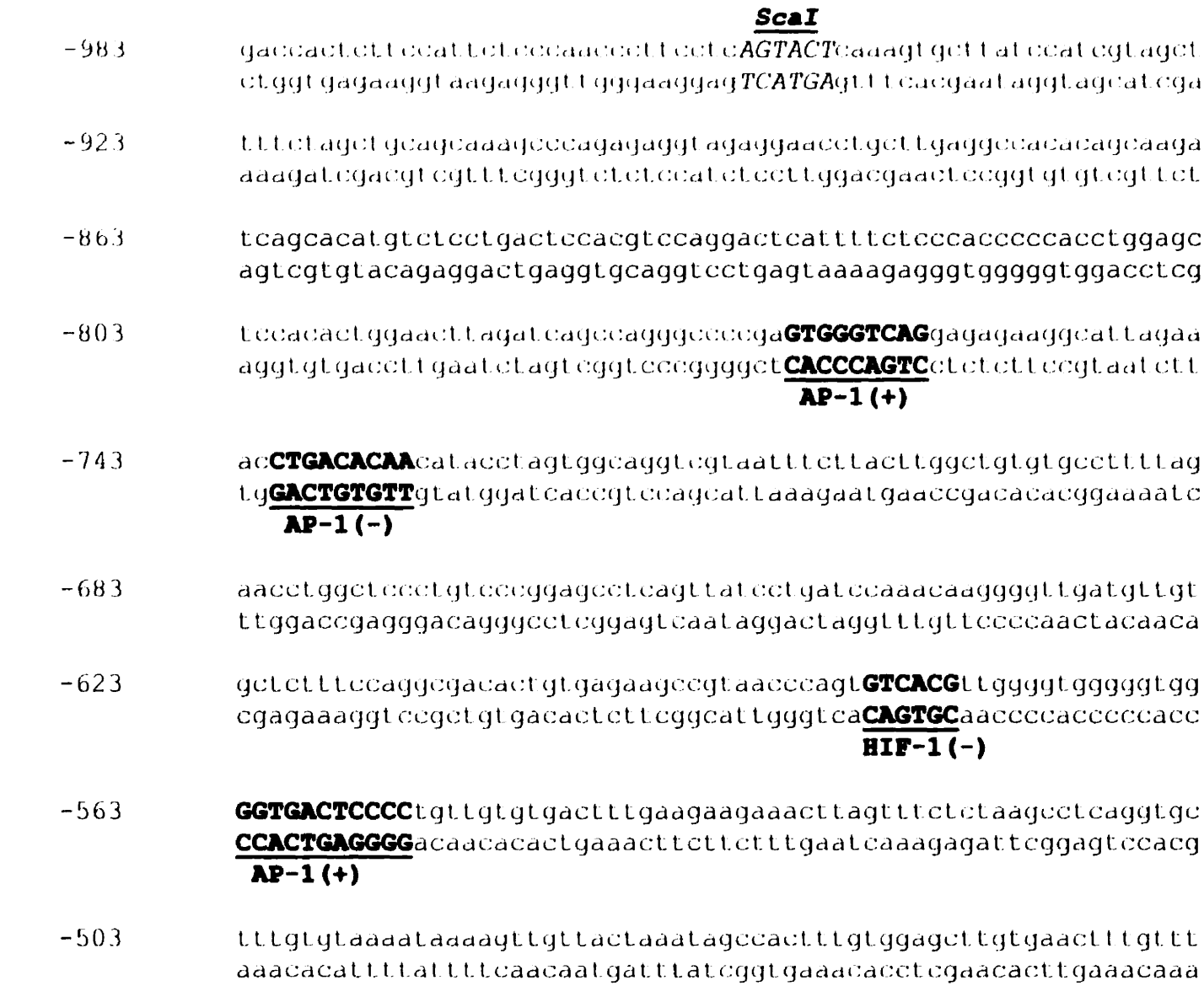

(bp)
EcoR V
Sca I
Dra I
Pvu II
Ssp I I
3054
2036
1636
1018
506

Figure 4.10

5' Regulatory Sequence Upstream of Exon 1b

Sequence determined for a clone of rat nNOS corresponding to regulatory sequences of the 5'end of the gene, upstream of exon 1b. The location of putative binding sites for the transcription factors HIF-1 and AP-1 are indicated. (-) denotes the presence of the binding site on the (-) strand of DNA (3'-5'direction) while (+) denotes the putative binding on the (+) DNA strand (5'-3' direction).


-443
-383
PvuII
-323
CTGACTCCAG
CTGAGCCA
GACTGAGGTC
GACTCGGT
AP-1 (-)
AP-1 (-)
-263
Exon 1c
-203
-143
Intron
-83
cgtgc
gcacg
HIF-1 (+)
-23
TGAGTC
ACTCAG
Exon 1b
AP-1 (+)
-38


ScaI
-983
gaccactctttccattctccccaaccctttcctcAGTACTcaaagtgcttatccatcgtagct
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AP-1(+)
-503
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DraI

-1283 gttaa*TTTAAA*aggagatgcaccttaatttctgtctccacagggtcggcgccccctaggat
caatt*AAATTT*tcctctacgtggaattaaagacagaggtgtccccagccgcggggatcctta

-1223 gtctgcccttaaatccctgcacacagtagatgttcaacaagtaccatttacc**CAGAGTCAG**
cagacgggaatttagggacgtgtgtcatctacaagttgttcatggtaatgg**GTCTCAGTC**
AP-1(+,-)

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-1043 ccagcagtgaggagagccgagcaggcccagggcacagggaaggatgccccagggagagtggg
ggtcgtcactcctctcgctcgtccgggtcccgtgtcccttcctacgggtcccctctcaccc

4.6 Discussion

We have previously demonstrated that the exposure of rats to prolonged hypoxia *in vivo* impairs contractility in response to adrenoreceptor stimulation in both resistance [1] and conductance [2] vessels (aorta). This loss in vascular contractility was shown to be endothelium-independent, and to develop within the first 12 hours of hypoxia, progress over the ensuing 48 hours and persist for at least 12 hours after the restoration of normoxia [2]. The production of NO in smooth muscle has been previously associated with vascular hypocontractility. We evaluated the possibility that either iNOS or nNOS may be upregulated in vascular smooth muscle during hypoxia since under certain circumstances, the expression of these isoforms has been reported to be inducible in vascular smooth muscle [6-9] and under the regulatory influence of hypoxia in certain tissues [10-14,16,18].

The main findings of this study are that following prolonged exposure to hypoxia *in vivo*: 1) nNOS protein and mRNA are induced in the rat aorta while iNOS mRNA, protein or activity could not be detected; 2) the induction of nNOS *in vivo* is localized to the vascular smooth muscle component of the aorta and is also detected in rat and human aortic smooth muscle cells cultured under hypoxic conditions; 3) the inhibition of NOS activity with L-NAME reverses the hypoxia-induced hypocontractility of aortic rings; 4) the 5'UTR of hypoxia-inducible nNOS transcript in rat smooth muscle corresponds to the previously identified exon1b sequence of rat nNOS; and 5) the 5' regulatory sequence upstream of exon 1b of rat nNOS harbors putative HIF-1 and AP-1 binding sites. These results constitute the first evidence that nNOS is specifically induced in the smooth muscle of the systemic vasculature in response to physiologically relevant levels of hypoxia *in vivo*

and consequently impairs smooth muscle contractility in response to adrenoreceptor stimulation.

In the current study, L-NAME increased the sensitivity and tension generated by endothelium-denuded aortic rings from rats exposed to hypoxia during activation by PE providing the first evidence of a functional NOS in the medial layer of these vessels. Furthermore, NOS inhibition normalized the PE-induced contractile response of endothelium-intact rings from hypoxic rats demonstrating an important role for NO in the observed loss in vascular contractility following hypoxia. It is important to note however, that NOS inhibition in endothelium-denuded rings did not completely restore maximal tension to the level observed in endothelium-denuded rings from normoxic rats. This suggests that NO is not the only factor involved in mediating the observed loss in vascular tone. It is likely that the activities and levels of expression of proteins which modulate or comprise the smooth muscle contractile apparatus such as myosin light chain kinase (MLCK), myosin light chain phosphatase (MLCP), the thin and thick myofilaments as well as their associated proteins may also be altered. Moreover, our observation that responses to PE for endothelium-intact aortic rings from hypoxic rats were normalized by L-NAME indicates that endothelium-derived constrictors are required for full restoration of contractility by NOS inhibition during hypoxia. In a previous study, we demonstrated that hypoxia causes an increase in endothelium-derived endothelin-1 (ET-1) release, which in turn accounts for the observed endothelial enhancement of smooth muscle contraction following hypoxia.

We have recently reported that, following exposure to hypoxia, the Ca^{2+}-dependent endothelial nitric oxide synthase (eNOS) protein and mRNA are decreased in the systemic

vasculature [27]. The absence of Ca^{2+}-independent NOS activity and the lack of any change in aortic Ca^{2+}/CaM-dependent NOS activity observed in the current study therefore indicated the concomitant increase of the other Ca^{2+}-dependent isoform, nNOS. This specific induction of nNOS in the aorta of hypoxic rats was confirmed by western analysis and localized to the vascular smooth muscle by immunohistochemistry. While some nNOS protein could be detected in aorta of normoxic rats, L-NAME did not influence the contractile response of endothelium-denuded rings from these rats. This may be due to the expression of nNOS in vessels from normoxic rats being predominantly localized to non-smooth muscle cells in the adventitial layer of the aorta that do not influence or participate in contraction (Figure 4.5). Our observations that nNOS protein and mRNA are increased in rat aortic smooth muscle cells following exposure to hypoxia supports our contention that this response is not due to changes in mechanical or neurohumoral stimuli to which these cells are exposed *in vivo*. Furthermore, the conservation of this response in both human and rat aortic smooth muscle cells following hypoxic incubation suggests that an understanding of the functional significance of nNOS induction in the rat aorta may provide clues for the management of hypoxia-induced vascular dysregulation in humans.

The regulation of nNOS gene expression is characterized by the use of alternative promoters resulting in the transcription of different exon 1 transcripts spliced to a common exon 2 containing the translation initiation site ATG [5,41-43]. Three exon 1 variants have been identified in the rat namely exons 1a, 1b and 1c. We have demonstrated that only the exon1b transcript of nNOS is induced in cultured vascular smooth muscle cells following exposure to hypoxia. In aortic tissues, the exon 1b transcript was expressed in the aorta from both normoxic and hypoxic rats. This transcript has been previously shown to be

localized in embryonal cells [42] and therefore its detection in aorta from normoxic rats is likely due to its expression in undifferentiated embryonal cells. The current study, however, represents the first report of the involvement of exon 1b in hypoxia-mediated induction of nNOS in vascular smooth muscle. In general, the 5' untranslated region of an mRNA transcript plays an important role in regulating mRNA translation by assisting in the binding and stabilization of the mRNA transcript on the ribosome where translation of the central segment occurs [47]. It follows that during hypoxia, exon1b would play a role in increasing the likelihood of nNOS protein expression in vascular smooth muscle. This sequence may therefore have important regulatory regions that bind specific proteins during hypoxia in order to mediate these effects.

The specific induction of the exon 1b transcript of rat nNOS in vascular smooth muscle following exposure to hypoxia suggests that the upstream regulatory sequence likely harbors binding sites for transcription factors involved in the expression of hypoxia inducible genes. Cloning and sequence analysis of the genomic sequence upstream of exon 1b of nNOS revealed two putative binding sites for HIF-1 and six sites for AP-1. These transcription factors are known to regulate the expression of hypoxia-inducible genes [28,31,32,48,49] and may therefore also be involved in the hypoxia-mediated induction of nNOS in vascular smooth muscle. HIF-1 binding sites in the promoter of nNOS may not only serve to activate the expression of nNOS in response to hypoxia, but may also provide a means to coordinate the level of nNOS expression and NO availability. Recently, it has been demonstrated that NO inhibits the accumulation and binding affinity of HIF-1 and may thereby serve to limit the expression of nNOS at the transcriptional level. Any

conclusions on the importance of these binding sites during hypoxia, however, must await future studies toconfirm their functional roles.

Normally, contractile agonists such as phenylephrine and ET-1 activate smooth muscle contraction through IP_3 (inositol triphosphate)-mediated Ca^{2+} release [50], an enhancement of myofilament Ca^{2+} sensitivity [51], and to some extent, an increase in Ca^{2+} influx due to the activation of L-type Ca^{2+} channels [52]. The induction of nNOS in vascular smooth muscle following hypoxia and concomitant NO release likely results in the interference with each of these steps and thereby blunts the *in vitro* responsiveness of these vessels to adrenoreceptor stimulation. The NO/cyclic guanosine monophosphate (cGMP)-mediated vascular smooth muscle cell relaxation is mediated by both a reduction in intracellular calcium $[Ca^{2+}]_i$ and by the activation of myosin light chain phosphatase (MLCP), which reduces the sensitivity of the contractile apparatus to $[Ca^{2+}]$ [53-57]. The activation of protein kinase G (PKG) by the NO/cGMP pathway has been shown to inhibit agonist-evoked formation of phospholipase C (PLC) and phosphorylate the IP_3 receptor, which is proposed to reduce the potency of IP_3-mediated Ca^{2+} release from the sarcoplasmic reticulum. Furthermore, both NO and cGMP can reduce Ca^{2+} entry by inactivating L-type Ca^{2+} channel conductance, independent of changes in membrane potential [58,59]. NO and PKG have also been shown to increase the activity of certain K^+ channels [58-62], thus causing hyperpolarization and reducing intracellular calcium. Finally, the activation of PKG 1α by cGMP increases the activity of MLCP thereby reducing the sensitivity of the myofilaments to intracellular calcium. Recently, it was also demonstrated that PKG 1α binds the myosin binding subunit (MBS) of MLCP and targets the phosphatase activity of this enzyme to the smooth muscle cell contractile apparatus [63].

The induction of the Ca^{2+}-dependent nNOS in vascular smooth muscle following hypoxia may serve as a means to coordinate the activation of this enzyme to the initiation of smooth muscle contraction. In hindsight, the hypoxia-mediated induction of Ca^{2+}-dependent nNOS instead of the Ca^{2+}-independent iNOS permits a highly regulated mechanism of smooth muscle function. One would expect that a rise in intracellular Ca^{2+} would simultaneously activate both nNOS and MLCK, thus immediately regulating the signaling pathway for contraction. The regulation of nNOS activation by Ca^{2+}, however, is quite complex and depends on the subcellular localization of this enzyme. In neurons and skeletal muscle, nNOS localizes to the cell membrane and is regulated by local increases in $[Ca^{2+}]$ due to the activation of distinct Ca^{2+} channels. For instance, while nNOS activity is regulated by L-type voltage-dependent channel in the myenteric plexus [64], Ca^{2+} influx associated with the stimulation of acetylcholine and NMDA receptors activates nNOS in skeletal muscle and brain, respectively [65,66]. Although we have not actually determined the localization of nNOS in vascular smooth muscle, we speculate that it may also be localized to the plasma membrane. In skeletal muscle, nNOS associates with membrane bound α1-syntrophin [66], a component of the dystrophin complex, via its protein-protein interaction or PDZ domain [67]. Smooth muscle cells express both α1-syntrophin [68] and dystrophin [69] and the dystrophin complex is concentrated in caveoli [70-72], thus providing a potential membrane localized binding site for nNOS. AT-1 receptors on smooth muscle cells sequester into caveoli by binding caveolin-1 [73] during activation with Ang II. This would place these receptors into close proximity to the dystrophin complex and possibly nNOS. It has been recently demonstrated that, in SHR, nNOS is expressed in the vascular smooth

muscle component of the common carotid artery where it modulates angiotensin II-induced contractions [9].

Although our results demonstrate that nNOS plays a central role in mediating the impairment in smooth muscle contraction following exposure to hypoxia, it is difficult to predict the impact of this enzyme on vascular contractility in the intact organism during hypoxia. Reduced oxygen availability will limit NOS activity since molecular oxygen is an essential co-substrate for NO synthesis. Of the three NOS isoform, nNOS has the highest K_m (45 mmHg) [74] for oxygen suggesting that its activity will be the most compromised in our model of hypoxia where the arterial PO_2 range is 35-39 mm Hg. Furthermore, a majority of nNOS normally binds self-generated NO to form a ferrous-NO complex, which causes it to operate at a fraction of its maximum possible activity during steady state [75]. The rate of breakdown of this inhibitory complex was shown to be directly proportional to oxygen concentration [76] suggesting that more nNOS will be in the inactive state during hypoxia. This does not however, mean that nNOS does not contribute to the vascular changes that occur *in vivo*. Numerous studies have demonstrated an increase in superoxide O_2^- generation during hypoxia[77-82]. NO can readily react with O_2^- and form the very powerful oxidant, peroxynitrite *in vivo* [83-90]. Peroxynitrite can attack many types of biological molecules, thus altering overall cellular function and possibly contributing to the observed vascular dysregulation during hypoxia.

Despite its nomenclature and its initial characterization as a constitutive enzyme [91], it is now accepted that nNOS is not exclusively expressed in neural tissues and can be inducible under certain conditions. The results of the current study implicate this enzyme in yet another biological function, which is that of attenuating smooth muscle contraction

and vascular tone during hypoxia. Our results provide the first evidence that physiologically relevant levels of hypoxia induce nNOS in the systemic vasculature and that this response plays a central role in the observed hypocontractility of aortic rings following hypoxia.

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Chapter 5

Summary and Conclusion

We have previously demonstrated that the exposure of rats to normobaric hypoxia (FIO_2=10%) *in vivo* for 12 and 48 hours has profound effects on the regulation of resistance arteriolar tone [1] and arterial [2] contractility. The abnormalities in vascular function reported in these studies include: 1) impaired endothelium-dependent vasoregulation, 2) loss of arteriolar myogenic responsiveness and 3) impaired smooth muscle contraction in response to adrenoreceptor stimulation in both resistance and conductance vessels. These alterations in both endothelial and smooth muscle cell function develop within the first 12 hours of hypoxia, progress over the ensuing 48 hours and persist for at least 12 hours after the restoration of normoxia [2]. The overall aim of this thesis was to identify key vasoregulatory genes and their products that may account for the observed hypoxia-induced changes in aortic endothelial and smooth muscle function following hypoxia *in vivo*.

The studies presented in this thesis, with the exception of that outlined in chapter 4, do not include experiments designed to dissociate the direct effects of hypoxia from those due to increased flow or to neurohumoral mediators released as part of the systemic vascular response. Nonetheless, hypoxia, as presented in these studies, simulates a clinically and physiologically relevant condition. The aorta was used in all of the studies presented in this thesis because this vessel provides sufficient mRNA and protein for all of the biochemical assays and, unlike arterioles, it can be easily manipulated for organ bath contractility experiments. The combination of the biochemical assays with *in vitro* contractility experiments provided a powerful tool in attempting to elucidate the role of specific vasoregulatory genes in mediating the circulatory responses to hypoxia.

The following is a summary of each of the studies presented in this thesis with emphasis on their original contributions to the field.

5.1. Endothelium

Normally, the endothelium exerts a tonic inhibitory effect on vascular reactivity, and agonist-induced contraction is greater in arterial segments from which the endothelium has been removed compared to those in which it is intact [3]. Following prolonged hypoxia however, we have demonstrated that endothelial ablation results in a decrease instead of an increase in the contractile response of aortic rings to phenylephrine [2]. This indicates that hypoxia causes a reversal in endothelial function such that the vascular endothelium serves as a source of substances that enhance rather than inhibit the contraction of the underlying smooth muscle. Although the inhibitory effect of the normal endothelium on vascular reactivity has been mainly attributed to the co-release of endothelial nitric oxide (NO) and vasodilatory prostaglandins [3,4], a decreased capacity for vasodilator synthesis, alone, cannot account for the observed endothelial enhancement of contractility. We therefore proposed that an increase in endothelium-derived constricting factor release must also occur concomitantly. The studies presented in chapters 2 and 3 provide a mechanistic explanation for our initial finding. In these chapters, we have demonstrated that hypoxia causes a profound downregulation of the endothelial nitric oxide synthase (eNOS) while increasing the production of endothelium-derived endothelin-1 (ET-1).

The study presented in chapter 2 is the first demonstration that physiologically relevant levels of hypoxia *in vivo* reduce eNOS expression in the systemic vasculature and consequently impair endothelium-dependent vascular responses. We analyzed the integrity of each step of the NO / cyclic guanosine monophosphate (cGMP) pathway in the aorta, beginning from eNOS activation at the level of the endothelial cell plasma membrane to the generation of cGMP via the activation of soluble guanylate cyclase in

vascular smooth muscle. We found that the abnormality in endothelium-dependent relaxation following *in vivo* hypoxia is not due to the inability to activate eNOS or to respond to its product (NO), but rather to a reduction in the availability of eNOS. While we expected basal aortic NO (measured as NO_3) and cGMP levels to be reduced following hypoxia, they were both unchanged. A likely explanation for these observations is the induction of neuronal nitric oxide synthase (nNOS, described in chapter 4) and hemoxygenase-1 (HO-1) [5] activity in vascular smooth muscle following hypoxia. NO derived from smooth muscle nNOS would increase both aortic NO_3 and cGMP levels, and the synthesis of carbon monoxide by HO-1 would activate soluble guanylate cyclase to also increase aortic cGMP [6,7]. It is thus not surprising that in aortic rings from rats exposed to hypoxia and subsequently stimulated with ACh, both cGMP and NO_3 levels were reduced compared to those observed in normoxic rats since ACh specifically activates eNOS in the endothelium and thus minimizes the contribution of other factors.

Endothelial release of nitric oxide (NO) has been shown to modulate myogenic autoregulatory responses [8] and to mediate active hyperemia, reactive hyperemia [9] and, in some vascular beds, hypoxic vasodilation [10]. A disruption in local NO production would therefore compromise these regulatory mechanisms that are of central importance in matching oxygen supply to metabolic demand and, therefore with the persistence of hypoxia, may contribute to organ system dysfunction. Furthermore, it has been recently shown that, under physiological conditions, eNOS-derived NO plays an important regulatory role in mitochondrial respiration and O_2 consumption [11]. NO inhibits mitochondrial respiration by nitrosylating the iron-sulfur centers of aconitase, complexes I and II of the electron transport chain, and through a reversible process, altering the

activity of cytochrome c oxidase [12-14]. A reduction in NO release during hypoxia would therefore have maladaptive effects since it would lead to an increase in O_2 consumption and demand in face of a reduction in O_2 delivery. This would worsen the oxygen deficit and further contribute to multi-organ system dysfunction. It is possible that a downregulation in eNOS may also impede normal angiogenesis since vascular endothelial growth factor (VEGF)-induced proliferation of endothelial cells has been shown to be NO-dependent. Finally, it is important to note that a reduction in eNOS expression and basal NO release below normal would also set the stage for an adaptive process to take place during hypoxia. The synthesis and activity of the constrictors, ET-1 [15,16] and TXA_2 [17,18]are known to be under the negative regulatory influence of NO. Accordingly, hypoxic inhibition of eNOS expression may play an additional role in the alteration in endothelial function through the removal of an inhibitor of vasoconstrictor production and activity. We have indeed demonstrated in chapter 3 that the release of ET-1 from the vascular endothelium accounts for the endothelial enhancement of aortic contractility following hypoxia.

The aim of the study presented in chapter 3 was to test the involvement of ET-1 and TXA_2 in hypoxia-induced endothelial enhancement of smooth muscle contraction. Our results confirm previous demonstrations of increased plasma ET-1 levels following hypoxia and provide the first direct evidence that survivable levels of hypoxia *in vivo* induce the expression of ET-1 protein and mRNA in the systemic vasculature. Interestingly, the ET_A receptor-specific antagonist, BQ123, mimicked the effect of removing the endothelium on maximum tension generated by aortic rings during activation by phenylephrine. Moreover, although smooth muscle cells are known to secrete ET-1 under certain conditions, the rise in aortic ET-1 was localized only to the

endothelium. This study provides conclusive evidence that ET-1 is the main mediator of the observed endothelial enhancement of smooth muscle contraction following hypoxia and that TXA_2 does not participate in this response. Interestingly, however, treatment of aortic rings with the TxA_2 receptor antagonist, SQ29548, reduced the maximum tension to PE-induced contraction in the normoxic group but did not have any effect in the hypoxic group. To our knowledge, this is a novel observation and suggests that alterations in the TxA_2 pathway may be yet another mechanism that contributes to the vascular dysregulation following hypoxia. NO is known to inhibit the activity and synthesis of TxA_2 and therefore, it is possible that smooth muscle nNOS-derived NO may play such an inhibitory role.

The synthesis of ET-1 is negatively regulated by nitric oxide[15]. The upstream 5'-flanking region of the ET-1 gene harbors response elements for the transcription factors AP-1 [19] and HIF-1[20] both of which are known to regulate the transcriptional activation of this gene during hypoxia. Recently, it has been shown that NO inhibits the binding of HIF-1 to its response elements [21]. In our model, the reduction of eNOS and decreased NO production in endothelial cells would therefore likely promote ET-1 transcription in the endothelium.

Based on our current understanding of ET-1 expression and its effects during systemic hypoxia, a central role in mediating the adaptive responses to systemic hypoxia is emerging for this molecule. In the lung, ET-1-mediated hypoxia vasoconstriction redistributes blood flow to well ventilated regions, thus optimizing the relationship between ventilation and perfusion[22,23]. ET-1 is also known to modulate the sensitivity of peripheral chemoreceptors[24,25] and thus regulates increases in ventilatory drive necessary to minimize the effects of reduced arterial oxygen tension. It is known that ET-1

activates the synthesis of vascular endothelial growth factor[26], which initiates angiogenesis and the formation of capillary networks needed to maximize oxygen extraction during hypoxia. Our results implicate ET-1 in yet another adaptive response to hypoxia, where its synthesis is upregulated in the vascular endothelium and acts locally on smooth muscle to limit the loss in adrenoreceptor vasoreactivity, which develops during hypoxia.

In summary, hypoxia induces the endothelium to become a source of substances, which enhance rather than inhibit contraction of the underlying smooth muscle. Despite the significant loss in smooth muscle contractility, we have previously shown that this important mechanism preserves responses to adrenoreceptor stimulation as well as pressure-sensitive myogenic responses in endothelium-intact diaphragmatic arterioles. In the studies presented in chapters 2 and 3, we have demonstrated that the hypoxia-induced endothelial enhancement of smooth muscle reactivity is mediated by the reciprocal regulation of eNOS and ET-1 in the vascular endothelium. While hypoxia regulates these genes independently, the reduction in eNOS expression and NO release also potentiates the hypoxia-mediated increase in ET-1 synthesis in the endothelium. As a result, these changes create an ideal microenvironment for endothelium-derived ET-1 to act locally on vascular smooth muscle and thereby enhance contraction. Overall, this adaptive mechanism acts to preserve sympathetic reflexes, and to delay the failure of blood flow autoregulation and the destabilization of transcapillary fluid flux at the expense of normal endothelium-dependent dilation.

5.2 Smooth muscle

Arteriolar myogenic reactivity determines the capacity to autoregulate blood flow and transcapillary fluid flux [27,28]. Furthermore, adrenergically-mediated reflexes affect the distribution of blood flow towards vital organs and the optimization of parenchymal blood flow in order to maximize oxygen extraction[29-32]. The preservation of smooth muscle reactivity is therefore necessary to prevent the disruption of vascular responses which maintain adequate tissue oxygen supply to vital organs. We have previously demonstrated that prolonged hypoxia causes a loss in myogenic arteriolar responsiveness and inhibits the contraction of smooth muscle in response to adrenoreceptor stimulation in both resistance[1] and conductance vessels[2]. This loss in smooth muscle contractility is endothelium-independent and is likely due to a change in gene expression in vascular smooth muscle since it persists for at least 12 hours following the restoration of normoxia. It follows that this alteration is most likely manifested by vasoregulatory genes whose products are contractile proteins and/or proteins that interfere with normal contraction. In chapter 4, we tested the inducible enzyme hypothesis by exploring the possibility that hypoxia may induce the expression of either iNOS and/or nNOS in vascular smooth muscle following hypoxia.

The study presented in chapter 4 is the first demonstration that physiologically relevant levels of hypoxia *in vivo* induce nNOS in the smooth muscle component of the systemic vasculature, and consequently impair contractile responses to adrenoreceptor stimulation. Our findings that hypoxia also elicits nNOS expression in cultured smooth muscle cells suggests that this is not due to changes in the mechanical or neurohumoral stimuli to which cells are exposed to *in vivo*. iNOS could not be detected in aorta from rats exposed to normoxia or hypoxia indicating that it is not involved in the vascular

responses to hypoxia. We also sequenced the upstream 5'regulatory sequences of nNOS in order to possibly gain some preliminary information on its regulation by hypoxia. Our results constitute the first report of the previously described exon 1b 5'UTR of nNOS [33] being expressed in vascular smooth muscle cells following hypoxia. We also show that the genomic sequence upstream of this exon harbors putative binding sites for transcription factors involved in hypoxic gene expression including HIF-1 and AP-1.

The induction of the Ca^{2+}/CaM-dependent nNOS in vascular smooth is likely to have profound effects on key mechanisms governing vascular tone. It is well known that the principal mechanisms that initiate contraction and relaxation in vascular smooth muscle are the rise and fall in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), respectively. Normally, an increase in smooth muscle $[Ca^{2+}]_i$ activates the Ca^{2+}/CaM-dependent MLCK which, in turn, phosphorylates the regulatory light chain of myosin (LC_{20}) and thus initiates contraction. The dephosphorylation of LC_{20}, on the other hand, is mediated by MLCP, which acts to oppose the actions of MLCK. The extent of LC_{20} phosphorylation and, therefore contraction is governed by the competing activities of both MLCK and MLCP [34]. Agonist-induced contractions increase intracellular calcium in smooth muscle and during hypoxia, would also activate nNOS to produce NO, which would in turn counteract the initial rise in calcium. This negative feedback loop would not only reduce the activity of nNOS, but would also likely interfere with smooth muscle contraction. A decrease in $[Ca^{2+}]_i$ would likely lead to the inactivation of MLCK and increased activation of MLCP, thus blunting the responsiveness of vascular smooth muscle to contractile agonists. Phenylephrine activates smooth muscle contraction through IP_3-mediated Ca^{2+} release [35] and enhancement of myofilament Ca^{2+} sensitivity [36] with a small effect on Ca^{2+} entry [37].

In chapter 4, we demonstrated that L-NAME enhanced the contractile response to phenylephrine in de-endothelialized aortic rings from hypoxic rats while having no effect in rings from normoxic rats. As expected, no effect was observed in aortic rings from normoxic rats. It is important to note however, that NOS inhibition in endothelium-denuded rings did not completely restore maximal tension to the level observed in endothelium-denuded rings from normoxic rats. This suggests that NO is not the only factor involved in mediating the observed loss in vascular tone. It is likely that the activities and levels of expression of proteins, which modulate or comprise the smooth muscle contractile apparatus such as MLCK, MLCP, the thin and thick myofilaments as well as their associated proteins, may also be altered. Moreover, our observations that responses to PE for endothelium-intact aortic rings from hypoxic rats were normalized by L-NAME indicate that endothelium-derived constrictors are required for full restoration of contractility by NOS inhibition during hypoxia. In a previous study, we demonstrated that hypoxia causes an increase in endothelium-derived endothelin-1 (ET-1) release, which in turn accounts for the observed endothelial enhancement of smooth muscle contraction following hypoxia.

The direct effects of NO may also mediate the nNOS-induced smooth muscle hypocontractility since this compound is a free radical that can readily interact with cellular proteins and inhibit their normal function. The fact that L-NAME did not completely reverse smooth muscle hypocontractility suggests the possibility that there may be a percentage of susceptible proteins (possibly involved in regulating contractile status) that may be irreversibly nitrated *a priori*. With the restoration of normoxia and the disappearance of nNOS, protein turnover would be expected to restore normal cellular function. Finally, the concentration of NO within a microenvironment decreases as the

distance between its point of origin and its target molecules increases. The induction of nNOS in vascular smooth muscle brings this enzyme in very close proximity to its target molecules so that even a reduction in its activation, possibly due to reduced O_2 concentration during hypoxia, may not reduce the overall potency of nNOS-derived NO.

With respect to the regulation of nNOS gene expression during hypoxia, we are the first to report that the promoter of rat nNOS harbors two putative HIF-1 binding elements. Furthermore, we have also identified multiple AP-1 binding regions within this sequence. The induction of nNOS in vascular smooth muscle may have profound effects on the mechanisms regulating intracellular calcium metabolism, contractile protein activation, and possibly negative regulatory feedback inhibition of nNOS expression and activation. NO has been shown to interfere with the activity of HIF-1 and therefore, its production in vascular smooth muscle may act to reduce nNOS mRNA levels through negative feedback inhibition. Although an attractive model, the functionality of these elements in nNOS induction during hypoxia has not yet been elucidated.

Future experiments should focus on whether or not exon 1b confers nNOS mRNA stability during hypoxia, thus increasing the likelihood of the translation of this mRNA in vascular smooth muscle. We have isolated and sequenced approximately 1.2 kb of DNA upstream of exon 1b in an attempt to identify regulatory elements that may provide clues in our understanding of hypoxia-mediated nNOS induction in vascular smooth muscle. Future luciferase studies with this promoter would identify the regions of DNA that are important in nNOS transcription. These regions could then be tested for the formation of protein complexes by electrophoretic mobility shift assays. These regions could also be used as probes to isolate novel transcription factors involved in hypoxia-mediated gene expression.

While the induction of nNOS may be a maladaptive vascular response with regards to contractility, its expression may also play a role in other adaptive responses. It is possible that nNOS is implicated in the prevention of smooth muscle cell proliferation in response to endothelium-derived increase in ET-1 and decrease in NO. This inhibition would, to some extent, preserve the contractile phenotype of vascular smooth muscle and thus prevent the further deterioration of smooth muscle contractility that is likely to occur with the appearance of the synthetic or proliferative smooth muscle cell phenotype.

Our study provides novel and exciting data which demonstrate that nNOS significantly contributes to the observed smooth muscle hypocontractility following hypoxia. From our perspective, with regards to the control of vascular tone, the induction of nNOS in vascular smooth muscle is maladaptive since it potentially impairs important autoregulatory responses for the optimization of flow and tissue oxygenation. At the expense of interfering with these regulatory responses, however, nNOS may simultaneously participate in other adaptive processes such as the preservation of smooth muscle contractile phenotype to promote the overall survival of the organism.

Hypoxia is a common clinical problem that frequently occurs in patients with pneumonia, congestive heart failure, shock and other cardiopulmonary diseases. We believe that the findings presented in this thesis significantly contribute to the advancement of our current understanding of the local factors involved in mediating overall changes in vascular function during hypoxia and may provide insight into the design of specific therapeutics geared towards the maintenance of vital organ integrity in patients suffering from dysregulation of vascular function.

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Statement of Originality

The following is a summary of the novel contributions made to the field of vascular biology/physiology by each of the studies presented in this thesis.

The study presented in chapter 2 is the first demonstration that physiologically relevant levels of hypoxia *in vivo* reduce eNOS expression in the systemic vasculature and consequently impair endothelium-dependent vascular responses. Previous studies on the regulation of eNOS by hypoxia have been done on endothelial cell cultures and have yielded conflicting results. Although it has been previously demonstrated that eNOS is upregulated in the lung following hypoxia for 7 days *in vivo*, the expression of eNOS in the systemic vasculature during these conditions had not yet been addressed. This study analyzed the integrity of each step of the NO / cyclic guanosine monophosphate (cGMP) pathway in the aorta, beginning from eNOS activation at the level of the endothelial cell plasma membrane to the generation of cGMP via the activation of soluble guanylate cyclase in vascular smooth muscle. It was found that the abnormality in endothelium-dependent relaxation following *in vivo* hypoxia is not due to the inability to activate eNOS or to respond to its product (NO), but rather to a reduction in the availability of eNOS. Furthermore, this study reproduced the previously observed upregulation of eNOS expression in the lung and thereby provided further support that eNOS expression is specifically reduced in the systemic circulation. Finally, our results suggest that eNOS downregulation is likely to contribute to the endothelial enhancement of α-agonist-induced contractions following hypoxia. These findings add considerably to our understanding of the effects of hypoxia on the regulation of eNOS expression in the

systemic circulation *in vivo* and its functional significance on endothelium-dependent responses.

The study presented in chapter 3 confirm previous demonstrations of increased plasma ET-1 levels following hypoxia and provide the first direct evidence that survivable levels of hypoxia *in vivo* induce the expression of ET-1 protein and mRNA in the systemic vasculature. Prior studies were unable to detect a rise in preproET-1 mRNA in the systemic vasculature following hypoxia *in vivo* and this may have been due to the use of Northern blotting, a relatively insensitive detection method for RNA. We circumvented this problem by conducting ribonuclease protection assays, a highly sensitive and quantitative technique. Although smooth muscle cells are known to secrete ET-1 under certain conditions, this study demonstrated that the rise in aortic ET-1 was localized only to the endothelium and provided conclusive evidence that endothelium-derived ET-1 is the main mediator of the observed endothelial enhancement of smooth muscle contraction following hypoxia. Our results implicate ET-1 in an adaptive response to hypoxia. ET-1 synthesis is upregulated in the vascular endothelium and acts locally on smooth muscle to limit the loss in adrenoreceptor vasoreactivity, which develops during hypoxia. While TxA_2 has been previously demonstrated to enhance ET-1 mediated contractions during conditions of reduced eNOS expression, our study showed that TxA_2 does not participate in this response following hypoxia. Interestingly, however, treatment of aortic rings with the TxA_2 receptor antagonist, SQ29548, reduced the maximum tension to PE-induced contraction in the normoxic group but did not have any effect in the hypoxic group. To our knowledge, this is a novel observation and

suggests that alterations in the TxA_2/PGH_2 pathway may be yet another mechanism that contributes to the vascular dysregulation following hypoxia.

The study presented in chapter 4 is the first demonstration that physiologically relevant levels of hypoxia *in vivo* induce nNOS in the smooth muscle component of the systemic vasculature, and consequently impair contractile responses to adrenoreceptor stimulation. Our findings that hypoxia also elicits nNOS expression in both rat and human cultured smooth muscle cells suggests that 1) nNOS induction is not due to changes in the mechanical or neurohumoral stimuli to which cells are exposed to *in vivo* and 2) this may be a conserved and important vascular alteration induced by hypoxia. Our results also constitute the first report that the previously described exon 1b 5'UTR of nNOS is expressed in vascular smooth muscle cells following hypoxia. Furthermore, this study demonstrated that the genomic sequence upstream of this exon harbors putative binding sites for important transcription factors involved in hypoxic gene expression namely, HIF-1 and AP-1. Overall, this study provides novel and exciting data which demonstrate that nNOS is a hypoxia-inducible gene in aortic vascular smooth muscle an its expression significantly contributes to the observed smooth muscle hypocontractility following hypoxia.