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

Mourad Toporsian, Karuthapillai Govindaraju, Mohammed Nagi, David Eidelman, Gaetan Thibault, Michael E. Ward

Abstract—The goal of this study was to determine whether hypoxia alters expression of endothelial nitric oxide synthase (eNOS) in the systemic circulation. Rats breathed either air or 10% oxygen for 12 hours, 48 hours, or 7 days. Thoracic aortas were 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^-3 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-cGMP were unaffected. Aortic cGMP levels after incubation with acetylcholine (10^-6 mol/L) averaged 14.0±1.8 fmol/mg in rings from normoxic rats compared with 8.7±1.0 fmol/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 for hypoxic rats and 7.8±0.7 μmol/L for normoxic rats). Impaired endothelial NO release may handicap the vascular responses that defend vital organ function during hypoxia. (Circ Res. 2000;86:671-675.)

Key Words: endothelium ■ systemic vasculature ■ hypoxic vasodilation ■ autoregulation

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 that redistribute the available oxygen supply toward vital organs and enhance oxygen extraction. Many of the mechanisms that 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 and to mediate flow dilation of resistance arterioles; active hyperemia; reactive hyperemia; and, in some vascular beds, hypoxic vasodilation. 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 that catalyzes NO synthesis, is regulated by oxygen tension, 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. 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 a change in eNOS availability alters vasoregulatory responses during hypoxia remains unanswered. Accordingly, the current study was undertaken to determine whether 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.

Materials and Methods

Male Sprague-Dawley rats were exposed to either normoxia or hypoxia (10% O2) for 12 hours, 48 hours, or 7 days.

Western Blot

Proteins from thoracic aorta and lung were analyzed using an eNOS-specific monoclonal antibody (Transduction Laboratories).

cDNA cloning

Aortic RNA was extracted using TRIzol. One microgram of RNA was reverse transcribed, and an 871-bp eNOS cDNA was amplified by polymerase chain reaction (PCR) using sense (5’-AGCTGGCATGGGCAACTTGAA-3’) and antisense (5’-CAGCACATCAAAGCGGCCATT-3’) primers and subcloned into the PCRII vector (Invitrogen) behind the T7 promoter (GenBank accession No. AF085195). β-Actin cDNA (292 bp) was also amplified by PCR (sense, 5’-AAGTACCCCCATTGAACACGGCA-...
3′; antisense, 5′-TAGATGGGACAGTGGGTTGA-3′) and sub-cloned into the PCRII vector (Invitrogen) behind the Sp6 promoter (GenBank accession No. AF122903).

32P-Labeled Riboprobe Synthesis and Ribonuclease Protection Assay
eNOS and β-actin constructs were linearized with BamHI and Bsa36I, respectively. Antisense RNA for eNOS and β-actin were synthesized using T7 and Sp6 RNA polymerases, respectively, from 1 µg of linearized construct and α-32P-labeled CTP (Amersham). The eNOS cRNA spanned regions homologous to exons 4 to 7 of the rat eNOS. The β-actin probe spanned exons 3 and 4 of the rat β-actin gene. Ribonuclease protection assays for eNOS and β-actin were carried out simultaneously on aortic RNA from each of the rats in each group. Protected mRNA was quantified by densitometry.

Endothelium-Dependent and -Independent Relaxation
Aortic segments (4 mm) from normoxic rats and rats exposed to hypoxia for 48 hours were treated with 10⁻³ mol/L phenylephrine. Concentration-response curves for acetylcholine (Ach; 10⁻⁸ to 10⁻⁴ mol/L) were generated, and maximum relaxations in response to A23187 (10⁻⁴ mol/L), diethylamine nitric oxide (DEA/NO, 10⁻⁴ mol/L), and 8-bromo-cGMP (8-Br-cGMP, 10⁻⁴ mol/L) were assessed.

cGMP Radioimmunoassay
Aortic segments (2 mm) from normoxic and 48-hour hypoxic rats were incubated at 37°C for 1 hour in Krebs solution, for 10 minutes in isobutylmethylxanthine (0.5 mmol/L), and then with Ach (10⁻⁶ mol/L) for 1 or 4 minutes or for 1 minute in the absence of Ach. The reaction was stopped with trichloroacetic acid (10% wt/vol). Trichloroacetic acid was removed with water-saturated ether, and the samples were acetylated with a 2:1 mixture of trifluoroethane and glacial acetic acid. The samples were incubated with a rabbit anti-rat cGMP antibody for 48 hours before overnight incubation with 125I-labeled cGMP. Rabbit serum and IgG were added, the samples were precipitated with 12.5% polyethylene glycol, and radioactivity was measured with a gamma counter.

Capillary Electrophoresis (CE)
Plasma nitrate (NO₃⁻) concentration was measured by CE. Plasma was collected from normoxic and 48-hour hypoxic rats, filtered (0.2 µm), and analyzed in a 50 mmol/L phosphate buffer (pH 2.5) with 0.5 mmol/L spermine using an ABI270 CE instrument with a 50-µm fused silica capillary (Polymicro Technologies). Samples were separated at 347 V cm⁻¹ (45 to 50 µA) at 30°C, and the NO₃⁻ peak was detected by absorption at 214 nm. Nitrate concentration was also measured in the reaction media from unstimulated and Ach-stimulated (4 minutes) aortic segments. Calibration curves were constructed with plasma or reaction media spiked with standard nitrate solutions.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results
eNOS Protein
Representative Western blots conducted on proteins from thoracic aortas and lungs from a normoxic rat and rats exposed to hypoxia for 12 hours, 48 hours, and 7 days are illustrated in Figures 1A and 1B, respectively. 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 versus normoxic controls) after 12 hours, 48 hours, and 7 days of hypoxia. Lung eNOS protein levels were unchanged after 12 and 48 hours of hypoxia but were increased (P<0.05 versus normoxic controls) after 7 days of hypoxia, as previously reported.

eNOS mRNA
A representative autoradiogram of an RNase 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 the top panel of Figure 2. The histogram in the lower panel illustrates the mean aortic eNOS mRNA levels in each group (n=4 per group) expressed as a percentage of the β-actin mRNA level. Aortic eNOS mRNA levels were decreased (P<0.01) after exposure to hypoxia for 12 hours, 48 hours, and 7 days compared with normoxic controls.

Endothelium-Dependent and -Independent Relaxation
The concentration-response relationships for Ach-induced relaxation in aortic rings from normoxic rats and from rats exposed to hypoxia for 48 hours are illustrated in the top panel of Figure 3. The tension generated during contraction with 10⁻³ mol/L phenylephrine was 1.67 ± 0.08 g/mg dry weight in rings from normoxic rats. As has been reported previously, 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 with 26.1±4.8% in rings from rats exposed to hypoxia (P<0.05 for difference). The pEC₅₀ 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 the bottom panel of Figure 3. 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, Ach 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).

cGMP Generation
The effect of hypoxia on aortic cGMP levels during in vitro stimulation with Ach is illustrated in Figure 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 Ach was lower (P<0.05) in the group exposed to hypoxia for 48 hours than in the normoxic group.

Plasma Nitrate Concentration and Aortic Nitrate Production
Concentrations of NO\textsubscript{3}\textsuperscript{−} in plasma from normoxic rats (n=7) and from rats exposed to hypoxia for 48 hours (n=6) averaged 70.3±2.6 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 that were not treated with Ach (control) and that were incubated with 10\textsuperscript{-6} mol/L Ach for 4 minutes are illustrated in Figure 5. Unstimulated values in the two groups did not differ. Nitrate concentrations were higher (P<0.05) after incubation with Ach in the normoxic than in the hypoxic group.

Discussion
The results of this study indicate that in rats, prolonged exposure to hypoxia results in (1) a decrease in aortic eNOS

Figure 2. A, Representative RNase 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 percentage of the β-actin mRNA level. **P<0.01 vs normoxic controls. Data are mean±SEM.

Figure 3. A, Concentration-response relationships for Ach-induced relaxation 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. B, Relaxation by A23187 (10\textsuperscript{-5} mol/L), DEA/NO (10\textsuperscript{-4} mol/L), and 8-br-cGMP (10\textsuperscript{-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. Data are mean±SEM.

Figure 4. cGMP levels in aortic segments from normoxic rats and rats exposed to hypoxia for 48 hours after incubation with Ach for 0, 1, and 4 minutes. *P<0.05 for difference between normoxic and hypoxic groups. Data are mean±SEM.
protein and mRNA, (2) impaired endothelium-dependent relaxation of phenylephrine-precontracted aortic rings, and (3) impaired capacity of aortic segments to generate cGMP and NO \textsubscript{3} in response to stimulation by Ach. 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, after exposure to hypoxia, relaxation in response to A23187 is decreased to a similar extent as relaxation to Ach points to an abnormality distal to events occurring at the level of the endothelial cell plasma membrane (ie, Ach receptor activation and Ca\textsuperscript{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-br-cGMP were not affected. The abnormality in endothelium-dependent relaxation after 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% \textsubscript{O}_{2} for 24 hours) decreased eNOS protein and mRNA in endothelial cells from human umbilical\textsuperscript{8} and saphenous\textsuperscript{15,16} veins and from bovine pulmonary artery\textsuperscript{9} and aorta.\textsuperscript{9,13} In contrast, Arnez et al\textsuperscript{10} reported that eNOS protein and mRNA were increased in bovine aortic endothelial cells after 24 hours of incubation at 1% \textsubscript{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 after hypoxic exposures of 30 to 240 minutes' duration.\textsuperscript{17} 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 that 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.\textsuperscript{18-20} 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,\textsuperscript{21} because inhibiting the increase in pulmonary blood flow by surgical stenosis of the pulmonary artery failed to prevent the increases in eNOS. Our present results confirm upregulation of lung eNOS protein after 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.\textsuperscript{1} 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 compose part of the response to systemic hypoxia, and the hypoxic exposures 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 with those in which it is intact.\textsuperscript{22,23} 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.\textsuperscript{23} After hypoxia, therefore, the endothelium serves as a source of substances that 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.\textsuperscript{22} 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\textsuperscript{12,14,25} and thromboxane A\textsubscript{2}\textsuperscript{26,27} 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\textsubscript{3} concentration. During hypoxia,
increased flow\(^1\) will stimulate NO synthesis by the remaining enzyme,\(^2,8\) the duration of eNOS activation may be increased as a result of increased pH of the endothelial intracellular space,\(^2,8,30\) and increased eNOS expression in the pulmonary circulation\(^18\) may counterbalance the effect of decreased NO synthesis in the systemic vasculature. Our inability to demonstrate a decrease in circulating NO\(^\text{--}\), 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\(^4\) and the decrease in transvascular resistance necessary to accommodate increased metabolic activity\(^5\) and to preserve vital organ perfusion during superimposed hypertensive stresses\(^3\) 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\(^7\) and canine diaphragm.\(^31\) In at least some essential vascular beds, therefore, this pathway must be intact 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. Because 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) of which the 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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**References**


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Methods

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

**Exposure to Hypoxia:** Rats were placed in a sealed plexiglas chamber (30 cm × 18 cm × 15 cm). Gas flow through the chamber was 6 L/min. 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 aortas and lungs were excised immediately after decapitation and frozen in liquid nitrogen for later extraction of protein or RNA. In separate experiments, thoracic aortas from normoxic rats and rats exposed to hypoxia for 48 hours were cut into segments and mounted in organ bath myographs for pharmacological studies.

**Western Blot Analysis:** The thoracic aortas 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 0.1 mmol/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 electrottransfer efficiency and was used as an indirect control for loading.
**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\(^1\) however, this region is conserved among the neuronal and inducible nitric oxide synthase isoforms as well as P450 reductases\(^1\). In order to provide a specific and quantitative assay of eNOS mRNA, therefore, 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 aortas 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\(^{TM}\) (Gibco BRL Life Technologies, Gaithersburg, MD) according to the method of Chomczynski and Sacchi\(^2\).

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. 1 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).

**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'\,-CAGCACATCAAAGCGGCCATT-3') primers. These primers were constructed based on the murine eNOS sequence\(^3\) after comparing this sequence with those encoding the murine neuronal\(^4\) and
inducible nitric oxide synthase isoforms for regions of least homology. The cDNA amplified using these primers was subcloned into the EcoRI site of pCR II vector (Invitrogen, San Diego, Ca.). DNA sequencing indicated 95% homology between this fragment and the murine eNOS mRNA sequence 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 (5’-AAGTACCCCATGGAACACCGGCA-3’) and antisense (5’-TAGATGGGCACAGTGTTGGGTGA-3’) primers and subcloned into the EcoRI site of PCR II vector (Invitrogen). DNA sequencing indicated that this cDNA corresponds to nucleotides spanning exon 3 and exon 4 of the rat β-actin gene 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 AF122903).

**P/rriboprobe 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-32P labeled CTP (400 Ci/mmol, Amersham, UK). The RNA polymerases, nucleotides and transcription buffer were provided with the Riboprobe 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 (Boehringer-Manheim, 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.

**Ribonuclease protection assay:** The ribonuclease (RNAase) protection assays were performed on RNA from the thoracic aortas 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 32P-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 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 phosphorimaging 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.

**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; Ca²⁺ 2.5; Mg²⁺ 1.2; Cl⁻ 151.3; HCO₃⁻ 25.0; SO₄²⁻ 1.2; H₂PO₄⁻ 1.4; dextrose 10), bubbled with 95% O₂, 5% CO₂ (pH = 7.4) at 37°C. Tension was adjusted to 2 g over 1 hour during which the buffer was replaced every 20 min. The rings were then contracted with 10⁻⁵M 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 (EC₅₀).

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} \text{ mol/L}) as above. The response to Ach (10^{-5} \text{ mol/L}) was assessed in each ring. The rings were then washed to remove these drugs, allowed to reequilibrate to their previous baseline tension of 2\text{g} and again contracted with phenylephrine (10^{-5} \text{ mol/L}). One of either A23187 (calcium ionophore, 10^{-5} \text{ mol/L}), diethylamine nitric oxide (DEA/NO, NO donor, 10^{-4} \text{ mol/L}) or 8-Bromo-cyclic guanosine monophosphate (8-Br-cGMP, cell permeable cGMP, 10^{-4} \text{ 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.

**Cyclic GMP Radioimmunoassay:** Thoracic aortas from normoxic rats (n=6) and rats exposed to hypoxia for 48 hours (n=9) were cut into 2 \text{ mm} segments which were randomly allocated to one of 3 groups. All were equilibrated for 1 hour in 4 \text{ mL} of Kreb's solution, pH 7.4, at 37°C then incubated for 10 \text{ min.} in isobutylmethylxanthine (0.5 \text{ mmol/L}). In two of the groups of segments, Ach (10^{-6} \text{ 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 \text{ minutes} was stored for measurement of nitrate concentration by capillary electrophoresis (see below).

The aortic segments were weighed and homogenized in 1 \text{ mL} 10\% TCA. The samples were centrifuged at 14,000 \times \text{ g} for 20 \text{ 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 CH₃COONa (0.35 mol/L), NaCl (0.15 mol/L), 2% BSA and 0.1% NaN₃. 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.

**Quantification of Nitrate by Capillary Electrophoresis:** Plasma nitrate ion (NO₃⁻) concentration was measured by capillary electrophoresis (CE) using a modification of previously described techniques. 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 electroosmotic 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 \(\mu\)A). These parameters allowed the detection of \(\text{NO}_3^-\) peak by ultraviolet absorption at 214 nm. This wavelength was chosen because chloride, which normally migrates in close proximity to \(\text{NO}_3^-\) and partially obscures its peak, does not absorb at 214 nm. The capillary oven temperature was at 30\(^\circ\) C during the analysis. All buffers were made fresh daily in doubly deionised water (Milli-Q unit; Millipore, Montreal, PQ, Canada) and filtered through 0.45 \(\mu\)m 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 \(\text{NaOH}\) (2 min.), with double deionized water (2 minutes.) 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 \(\text{NO}_3^-\) 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 \(\mu\)mol/L range.

The above CE parameters were also used to measure the concentration of \(\text{NO}_3^-\) in the reaction media from unstimulated aortic segments and from aortic segments which were incubated with \(\text{Ach}\) \((10^{-6}\) 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 \(\mu\)mol/L) and were linear \((r=0.996)\) over this range.

**Statistical Analysis:** Between groups comparisons 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.

Reference List


