ELSEVIER

European Journal of Pharmacology 421 (2001) 45–53

ejp

www.elsevier.nl/locate/ejphar

Hypoxia impairs endothelium-dependent relaxation in organ cultured pulmonary artery

Takahisa Murata*, Hideyuki Yamawaki, Masatoshi Hori, Koichi Sato, Hiroshi Ozaki, Hideaki Karaki

Department of Veterinary Pharmacology, Graduate School of Agriculture and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo, Tokyo 113-8657, Japan

Received 14 December 2000; received in revised form 18 April 2001; accepted 24 April 2001

Abstract

In intrapulmonary arteries cultured under hypoxic conditions (5% oxygen) for 7 days, endothelium-dependent relaxation and cGMP accumulation induced by substance P were decreased as compared to those of a normoxic control (20% oxygen). In rabbit mesenteric arteries exposed to chronic hypoxia, however, endothelial dysfunction was not observed. Furthermore, in endothelium-denuded pulmonary arteries exposed to hypoxia, neither relaxation nor cGMP accumulation due to sodium nitroprusside differed from those of the normoxic control. Hypoxia did not change the mRNA expression of endothelial NO synthase (eNOS), the protein expression of eNOS or the eNOS regulatory protein caveolin-1 as assessed by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) or whole-mount immunostaining. Morphological study revealed atrophy of endothelial cells and condensation of the eNOS protein in many cells. These results suggest that chronic hypoxia impaired NO-mediated arterial relaxation without changing either the eNOS protein expression or the NO-sensitivity of smooth muscle cells in pulmonary arteries. Changes in cell structure and organization may be involved in endothelial dysfunction.

Keywords: Hypoxia; Organ culture; Endothelium-dependent relaxation

1. Introduction

The vascular endothelium contributes to the local regulation of vascular smooth muscle tone by releasing various vasoactive products such as nitric oxide (NO) (Pollock et al., 1991). The enzyme responsible for NO production in the vascular endothelium is endothelial NO synthase (eNOS). NO is produced by eNOS using an amino acid, L-arginine, and molecular oxygen as substrates and other cofactors including NADPH and tetrahydrobiopterin (Griendling and Alexander, 1996). Additionally, effective NO production requires the interaction of eNOS and the cell membrane (Fulton et al., 1999). Above all, caveolin-1, a structural component of the cell membrane, is reported to regulate eNOS activity (Liu et al., 1996; Garcia-Cardena et al., 1997).

NO produced in the endothelium causes smooth muscle relaxation by the activation of soluble guanylate cyclase followed by cGMP accumulation to activate cGMP-dependent protein kinase (G kinase) (Gruetter et al., 1981). Although eNOS is expressed constitutively in endothelial cells, its gene expression is regulated by various stimuli such as shear stress, hemodynamics, and neuronumoral stimuli (Förstermann et al., 1995).

It has been reported that in pulmonary arteries isolated from patients with pulmonary hypertension due to chronic obstructive lung disease, the release of NO from the endothelium is impaired (Xue and Johns, 1995; Giaid and Saleh, 1995). Additionally, chronic hypoxia-induced pulmonary hypertensive rats exhibit a decrease in endothelium-dependent relaxation of pulmonary arteries (Shaul et al., 1993). These reports support the idea that chronic hypoxia impairs NO-dependent vasodilation in the pulmonary arteries, and induces pulmonary hypertension and right ventricular hypertrophy (Zapol et al., 1977; Zapol and Jones, 1987).

In some studies using cultured endothelial cells from human umbilical veins (McQuillan et al., 1994) and saphenous veins (Phelan and Faller, 1996) and from bovine pulmonary artery and aorta (Liao et al., 1995), hypoxic

* Corresponding author. Tel.: +81-3-5841-5393; fax: +81-3-5841-8183.

E-mail address: murata@jvm2.vm.a.u-tokyo.ac.jp (T. Murata).

0014-2999/01/$ - see front matter
PII: S0014-2999(01)01015-9

conditions (0% O_2 for 24 h) decreased NO production via suppression of eNOS mRNA and/or eNOS protein, which supports the hypothesis that chronic hypoxia decreases eNOS expression. However, Arnet et al. (1996) reported that eNOS protein and mRNA were increased in bovine aortic endothelial cells after 24 h of incubation at 1% O_2 (Arnet et al., 1996). Additionally, several in vivo studies have revealed that eNOS mRNA and/or protein expression are increased in pulmonary arteries from patients with pulmonary hypertension (Xue and Johns, 1995) and hypoxia-induced pulmonary hypertensive rats (Shaul et al., 1993). These results indicate that an impairment of endothelium-dependent relaxation may be induced by down-regulation of eNOS activity rather than suppression of eNOS expression.

Even if the previous data were consistent, the results of cell culture experiments are conflicting and reflect differences in the vascular beds from which the endothelial cells were derived, and the methods used to maintain the cells. However, in vivo studies cannot rule out the possibility that factors other than decreased O_2 tension, such as shear stress, hemodynamics, and neuronumoral factors, may change eNOS expression (Xiao et al., 1997; Förstermann et al., 1995). Therefore, at present, the role of eNOS in the pathogenesis of this disease remains controversial, and the mechanisms responsible for the loss of endothelium-dependent relaxation induced by chronic hypoxia have not yet been clarified.

In this study, we examined the changes in endothelial NO synthesis in rabbit pulmonary arteries cultured under a low oxygen tension for 7 days. The organ culture method makes it possible to dissociate the influence of other factors from the direct effect of hypoxia, to incubate the tissue under a constant oxygen tension for a long period of time, and to easily examine the tissue for both morphology and functional changes. Our results indicate that chronic hypoxia impairs endothelium-dependent relaxation without changing eNOS mRNA and protein expression, and specifically causes atrophy of endothelial cells in the pulmonary artery.

2. Materials and methods

2.1. Tissue preparation and organ culture procedure

Male Japanese White rabbits (2–3 kg) were euthanized by stunning and exsanguination. The organ culture procedure was performed as described previously (Yamawaki et al., 1999). Briefly, the main branches of the intrapulmonary arteries or the main branches of the superior mesenteric arteries were isolated. After fat and adventitia were removed in sterile Hanks' balanced salt solution, each artery was cut into rings (approximately 1.5 mm wide). The arterial rings were then placed in 2 ml of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% penicillin–streptomycin. The muscle rings were maintained at 37°C under an atmosphere of 90% N_2–5% O_2 and 5% CO_2 (hypoxia) or 95% air and 5% CO_2 (normoxia) for 7 days. The medium was changed every 2 days until the experiments were started. Animal care and treatment were conducted in conformity with the institutional guidelines of The University of Tokyo.

2.2. Measurement of muscle tension

In the experiments to examine the effect of chronic hypoxia on vascular smooth muscle, the endothelium was removed by gently rubbing the intimal surface with forceps after the organ culture procedure. The arterial rings with or without endothelium were placed in normal physiological salt solution (PSS) of the following composition (mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.0, $NaHCO_3$ 23.8, and glucose 5.5. Ethylenediaminetetraacetic acid (EDTA, 1 μM) was also added to remove contaminating heavy metal ions, which would have catalyzed the oxidation of organic chemicals. The high-K^+ solution (30 mM KCl) was prepared by replacing NaCl with equimolar KCl. The PSS was saturated with a 95% O_2, 5% CO_2 mixture at 37°C and pH 7.4. Muscle tension was recorded isometrically with a force-displacement transducer (Orientec, Japan) connected to a strain amplifier (Model 3134 or 3170, Yokogawa, Japan) and an ink-writing recorder (Model 3056 or 3711, Yokogawa, Japan) under a resting tension of 10 mN. Data are shown as percent relaxation of the steady-state preconstriction.

2.3. Measurement of cGMP content

Subsequent to incubation with or without the test agent, the vascular rings were immediately frozen in liquid nitrogen and homogenized in 6% trichloroacetic acid solution. After centrifugation at $2000 \times g$ for 15 min at 4°C, supernatants were applied to a cGMP enzyme-immunoassay system (Amersham, UK), whereas pellets were used to determine protein content by the method of Bradford (1976). cGMP content is expressed as pmol/mg protein content.

2.4. Measurement of eNOS mRNA expression

Total RNA was extracted from the arterial rings using the acid guanidinium thiocyanate–phenol–chloroform method with the TRIzol reagent. The concentration of total RNA was adjusted to 1 μg/μl with RNase-free distilled water. Reverse transcription-polymerase chain reaction (RT-PCR) was performed as described previously (Nakazawa et al., 1999). Briefly, the first strand of cDNA was synthesized using random 9-mers RT-primer and AMV Reverse Transcriptase XL at 30°C for 10 min, 55°C for 30 min, 99°C for 5 min, and 4°C for 5 min, followed by PCR amplification using synthetic gene-specific primers for

eNOS and GAPDH. PCR amplification was performed using Taq DNA polymerase (Ampli Taq Gold). The oligonucleotide primers for eNOS and GAPDH were designed as described previously (Nishida et al., 1992). The forward primers and reverse primers for endothelial NO synthase and GAPDH were designed as follows: ATA GAA TTC ACC AGC ACC TTT GGG AAT GGC GAT (forward primer for eNOS), ATA GAA TTC GGA TTC ACT GTC TGT GTT GCT GGA CTC CTT (reverse primer for eNOS), TCC CTC AAG ATT GTC AGC AA (forward primer for GAPDH), and AGA TCC ACA ACG GAT ACA TT (reverse primer for GAPDH). After denaturation at 95°C for 10 min, 27–39 cycles of amplification at 94°C for 0.4 min, 55°C for 1 min, and 72°C for 1.5 min were performed using a thermal cycler (Takara PCR Thermal Cycler MP, Takara Biomedicals, Japan). The PCR products were electrophoresed onto 2% agarose gel containing 0.1% ethidium bromide. The possibility of their containing DNA was excluded by a PCR with total RNA without the reverse transcription step. Electrophoresis was performed at 100 V for 40 min, and the detectable fluorescence bands were visualized using a UV-transilluminator. The densitometric intensity of 260 base pairs for eNOS and of 308 base pairs for GAPDH at 30 cycles was quantified using an image-processing program, NIH Image 1.55. The results are expressed as the ratio of the optical density of eNOS to that of GAPDH.

2.5. Measurement of eNOS and caveolin-1 protein expression and area of endothelial cells

To determine the amount of eNOS and caveolin-1 protein expression in endothelial cells, we applied the whole-mount immunostaining method. After incubation, the arterial rings were fixed with Zamboni's fixative. Fixed arteries were incubated with 0.3% Triton X-100 and 10% normal goat serum dissolved in phosphate-buffered saline (PBS) for 1 h and then probed with anti-eNOS monoclonal antibody (1:1000 dilution) or anti-caveolin-1 polyclonal antibody (1:500 dilution). The second reaction, using either fluorescein isothiocyanate (FITC)-labeled anti-mouse immunoglobulin G (Ig G) (1:500 dilution) or rhodamine-labeled anti-rabbit IgG (1:500 dilution), was then performed and viewed, and the images were captured with a Carl Zeiss confocal laser scanning microscope LSM510 imaging system ($\times 630$). The microscope was equipped with excitation (FITC: 458–488 nm, rhodamine: 543 nm) and emission (FITC: 520 nm, rhodamine: 570 nm) filters. To compare the amounts of protein expression, 15-μm-thick images from the endothelial cell surface were digitized under a constant exposure time, gain, and offset. After the images were captured, the densitometric intensity was quantified with NIH Image 1.55. A binary layer mask was applied to the defined unit area (1.96×10^4 μm^2), the intensity of each pixel (0 to 255 arbitrary units) in these areas was measured and the value of each mean intensity was obtained. Zero represented the lower limit of light detection and 255 the saturation level. Data are shown as arbitrary units (fluorescence intensity) per unit area.

Additionally, to measure the area of endothelial cells, the endothelium was labeled with a specific endothelial cell marker, acetylated low-density-lipoprotein labeled with 1,1′-dioctadecyl-3, 3, 3′, 3′-tetramethylindo-carbocyanine perchlorate (Dil-Ac-LDL). The arterial rings after the organ culture procedure were incubated with 10 μg/ml Dil-Ac-LDL at 37°C for 4 h, and the images of endothelial cells were captured with the confocal laser scanning microscope ($\times 630$) as mentioned above (excitation: 543 nm, emission: 570 nm). To compare the cell areas, we focused on the 5.2-μm-thick sections showing the widest area in each cell, and captured the image. The area of each cell (including nucleus without Dil-Ac-LDL labeling) was measured using NIH Image 1.55. Data are shown as μm^2/cell.

2.6. Chemicals

The chemicals used were as follows: prostaglandin $F_2\alpha$, substance P, ionomycin calcium salt (Sigma, USA), random 9-mers RT-primer (Takara Biomedicals, Japan), AMV Reverse Transcriptase XL (Life Science, USA), Hank's balanced salt solution, penicillin–streptomycin, TRIzol reagent, ethidium bromide solution (Gibco BRL, USA), DMEM (Nissui Pharmaceutical, Japan), Ampli Taq Gold (Roche, USA), anti-caveolin-1 polyclonal antibody (Santa Cruz Biotechnology, USA), anti-eNOS monoclonal antibodies (Transduction Laboratories, USA), and Dil-Ac-LDL (Biomedical Technologies, USA).

2.7. Statistical analysis

The results of the experiments are expressed as means ± S.E.M. Statistical evaluation of the data was performed using unpaired Student's *t*-test, and a value of $P < 0.05$ was taken as significant.

3. Results

3.1. Endothelium-dependent relaxation

In pulmonary arteries with endothelium cultured under normoxia or hypoxia for 7 days, the cumulative addition of prostaglandin $F_2\alpha$ (3 nM–10 μM) induced contractions with similar amplitudes (a maximum force of 46.3 ± 7.0 and 45.6 ± 2.6 mN/mg wet weight, respectively) in a concentration-dependent manner with an EC_{50} value of 293 ± 82 and 312 ± 79 nM, respectively ($n = 10$ each).

In the normoxic pulmonary arteries with endothelium, substance P (0.1–10 nM) caused vasorelaxation of the muscle contraction elicited by 1 μM prostaglandin $F_2\alpha$ in

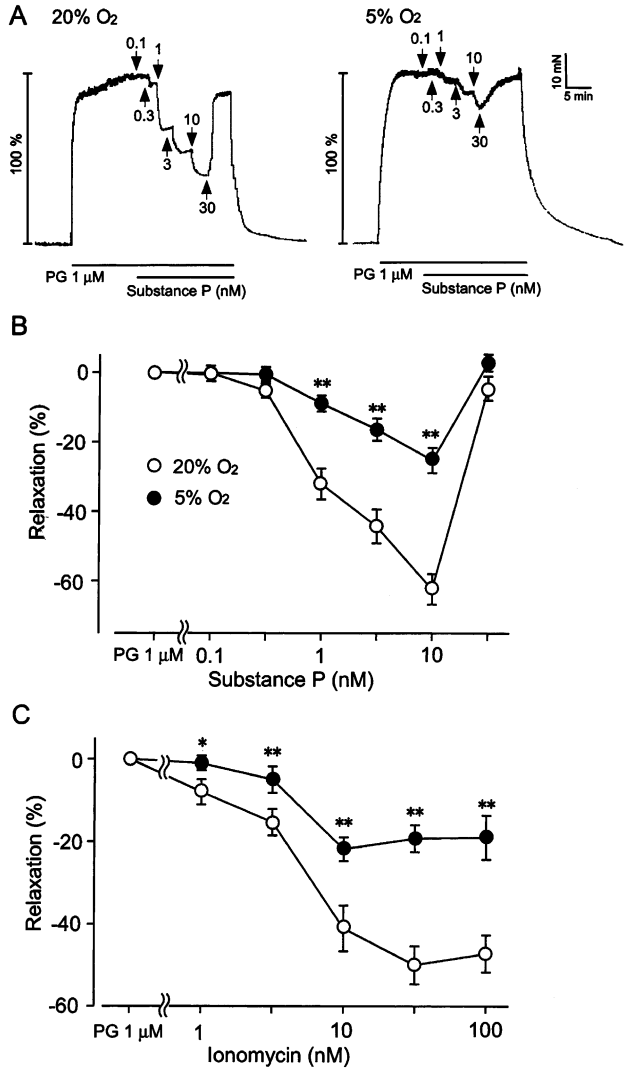

Fig. 1. Effects of substance P on prostaglandin $F_2\alpha$ (1 μM)-induced contraction in pulmonary artery cultured under normoxic (20% O_2) or hypoxic (5% O_2) conditions for 7 days (A: typical trace of relaxation by substance P; B: summary of the results). Effects of ionomycin on prostaglandin $F_2\alpha$ (PG) (1 μM)-induced contraction in the pulmonary artery cultured under normoxic (20% O_2) or hypoxic (5% O_2) conditions for 7 days (C). Substance P or ionomycin was added after the prostaglandin $F_2\alpha$-induced contraction had reached a steady-state level. Results are expressed as means ± S.E.M. ($n = 10$–15). * and **: significantly different from the normoxic arteries with $P < 0.05$ and $P < 0.01$, respectively.

a concentration-dependent manner. At a higher concentration (30 nM), substance P induced contraction, which may be due to a direct contractile effect on smooth muscle. In the hypoxic pulmonary arteries, the substance P-induced endothelium-dependent relaxation was significantly attenuated compared to that in the normoxic pulmonary arteries. The maximum relaxation due to substance P was 62.2 ± 3.5% ($n = 12$) in the normoxic arteries and 25.2 ± 3.7% ($n = 15$) in the hypoxic pulmonary arteries ($P < 0.01$; Fig. 1A and B). In the hypoxic pulmonary arteries, furthermore, the relaxation of the 30 mM high K^+-induced contraction by substance P (10 nM) was much smaller than that in the normoxic pulmonary arteries (the maximum relaxation due to substance P: 45.6 ± 2.6% ($n = 6$) in the normoxic arteries, 11.6 ± 4.2% ($n = 6$) in the hypoxic pulmonary arteries; $P < 0.01$).

In the normoxic pulmonary arteries with endothelium, ionomycin (1 nM–100 μM) caused vasorelaxation of the muscle contraction elicited by 1 μM prostaglandin $F_2\alpha$ in a concentration-dependent manner (Fig. 1C). The vasodilator effect of ionomycin was significantly attenuated in the hypoxic pulmonary arteries: the maximum relaxation due to ionomycin was 50.0 ± 5.6% ($n = 12$) in the normoxic pulmonary arteries and 21.8 ± 2.9% ($n = 11$) in the hypoxic pulmonary arteries ($P < 0.01$).

In the mesenteric arteries cultured under normoxia for 7 days, substance P (0.1–30 nM) caused vasorelaxation of the 1 μM noradrenaline-induced contraction in a concentration-dependent manner, and chronic hypoxia (for 7 days) did not change the substance P-induced relaxation (maximum relaxation, normoxia: 69.8 ± 2.5%, $n = 6$; hypoxia: 67.5 ± 2.7%, $n = 7$; Fig. 2A and B). Furthermore, chronic hypoxia did not change the relaxation induced by 10 nM substance P of the 30 mM high K^+-induced contraction (the maximum relaxation due to substance P: 49.6 ± 2.3% ($n = 6$) in the normoxic arteries, 46.8 ± 4.2% ($n = 6$) in the hypoxic pulmonary arteries).

In all of the arteries without endothelium, substance P was ineffective. The substance P (100 nM)-induced relaxation was abolished by treatment with N^G-monomethyl-L-

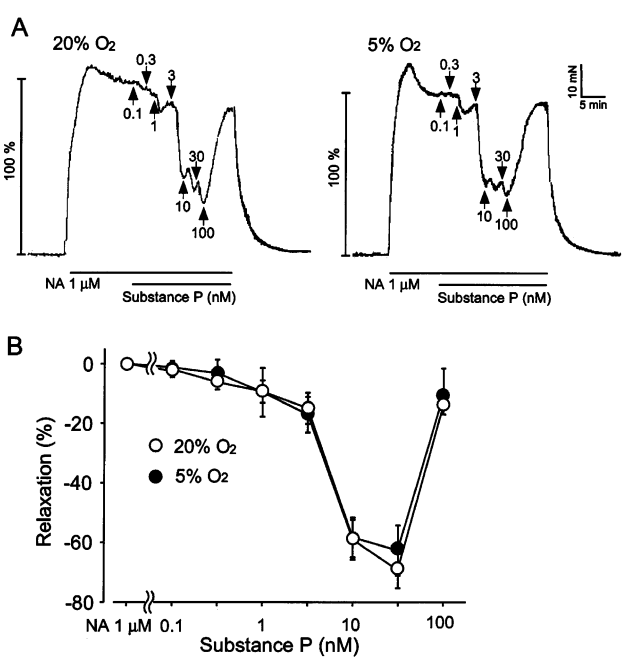

Fig. 2. Effects of substance P on noradrenaline (NA) (1 μM)-induced contraction in mesenteric artery cultured under normoxic (20% O_2) or hypoxic (5% O_2) conditions for 7 days (A: typical trace of relaxation by substance P; B: summary of the results). Substance P was added after the noradrenaline-induced contraction had reached a steady-state level. Results are expressed as means ± S.E.M. ($n = 6$–7).

arginine (L-NMMA), a NOS inhibitor (200 μM, 30 min before the addition of prostaglandin $F_2\alpha$ or noradrenaline) ($n = 6$ each; data not shown). Treatment with indomethacin, a cyclooxygenase inhibitor (10 μM, 30 min before the addition of prostaglandin $F_2\alpha$ or noradrenaline), did not change the endothelium-dependent relaxation caused by substance P ($n = 6$ each; data not shown). These results suggest that the relaxation of the cultured arteries is attributable mainly to NO production.

3.2. Sodium nitroprusside-induced relaxation

In the normoxic and hypoxic pulmonary arteries without endothelium, sodium nitroprusside (1 nM–3 μM) caused vasorelaxation of the contractions elicited by 1 μM prostaglandin $F_2\alpha$ in a concentration-dependent manner. The maximum relaxation due to sodium nitroprusside was $89.6 \pm 3.3\%$ and $91.4 \pm 1.8\%$ in normoxic and hypoxic pulmonary arteries, respectively ($n = 6$ each; Fig. 3A).

3.3. Depletion of L-arginine, tetrahydrobiopterin, and the effect of superoxide dismutase

To determine whether the hypoxia-induced impairment of endothelium-dependent relaxation was caused by depletion of L-arginine, a precursor of NO (Eddahibi et al., 1992), or tetrahydrobiopterin, a cofactor for NO production (Maier et al., 2000), we examined the effect of each of these agents on the substance P-induced relaxation. Each preparation was treated with L-arginine (1 mM) and/or tetrahydrobiopterin (1 μM) for 20 min before the addition of 1 μM prostaglandin $F_2\alpha$, but these treatments did not change the 0.1 nM–10 nM substance P-induced relaxation: the maximum relaxation due to substance P was $25.9 \pm 3.1\%$ ($n = 9$) in non-treated hypoxic arteries, $24.8 \pm 2.6\%$ ($n = 7$) in the L-arginine-pretreated hypoxic arteries, $24.3 \pm 3.5\%$ in the tetrahydrobiopterin-pretreated hypoxic arteries ($n = 7$) and $25.2 \pm 3.8\%$ in the L-arginine and tetrahydrobiopterin pretreated hypoxic arteries ($n = 7$). We also examined the effect of superoxide dismutase, a superoxide scavenger, on the impairment of endothelium-dependent relaxation, because free radical superoxide anion can interact with NO and prevent its vasodilator activity (Rubanyi and Vanhoutte, 1986; Beckman et al., 1990). However, in the hypoxic arteries, the addition of superoxide dismutase (150 U/ml) (20 min before the addition of 1 μM prostaglandin $F_2\alpha$) did not change the 0.1 nM–10 nM substance P-induced relaxation: the maximum relaxation due to substance P was $26.8 \pm 4.2\%$ in the superoxide dismutase-pretreated hypoxic arteries ($n = 7$).

3.4. cGMP content

In the arteries with endothelium under resting condition, the cGMP content in the hypoxic pulmonary arteries did not differ from that in the normoxic pulmonary arteries (hypoxia: 47.1 ± 1.4 pmol/mg, normoxia: 36.3 ± 3.4 pmol/mg; $n = 5$, each; Fig. 3B). Sodium nitroprusside (1 μM) increased the cGMP content in a time-dependent manner in the pulmonary arteries without endothelium (data not shown), and a 3-min treatment with sodium nitroprusside (1 μM) increased the cGMP content maximally. No difference was found in the sodium nitroprusside-increased the cGMP content in the hypoxic and normoxic pulmonary arteries (hypoxia: 173.6 ± 3.1 pmol/mg, normoxia: 187.3 ± 3.5 pmol/mg; $n = 5$ each). In contrast, substance P (10 nM) increased the cGMP content in a time-dependent manner in the normoxic and hypoxic pulmonary arteries with endothelium (data not shown), and a 2-min treatment with substance P (10 nM) increased the

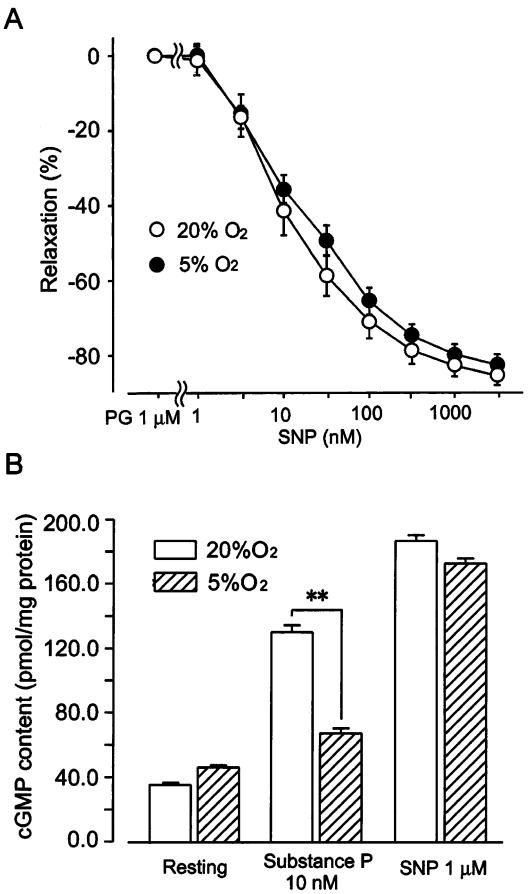

Fig. 3. Effects of sodium nitroprusside on the prostaglandin $F_2\alpha$ (PG) (1 μM)-induced contraction in pulmonary arteries without endothelium cultured under normoxic (20% O_2) or hypoxic (5% O_2) conditions for 7 days (A). Sodium nitroprusside (SNP) was added after the prostaglandin $F_2\alpha$-induced contraction had reached a steady-state level. Results are expressed as means ± S.E.M. ($n = 6$ each). Changes in cyclic GMP content induced by substance P and sodium nitroprusside in pulmonary artery cultured under normoxic (20% O_2) or hypoxic (5% O_2) conditions for 7 days (B). Endothelium-intact pulmonary arteries were treated with 10 nM of substance P for 2 min, and endothelium-denuded pulmonary arteries were treated with 1 μM sodium nitroprusside for 3 min. Results are expressed as means ± S.E.M. ($n = 5$ each). **: significantly different from the normoxic arteries with $P < 0.01$.

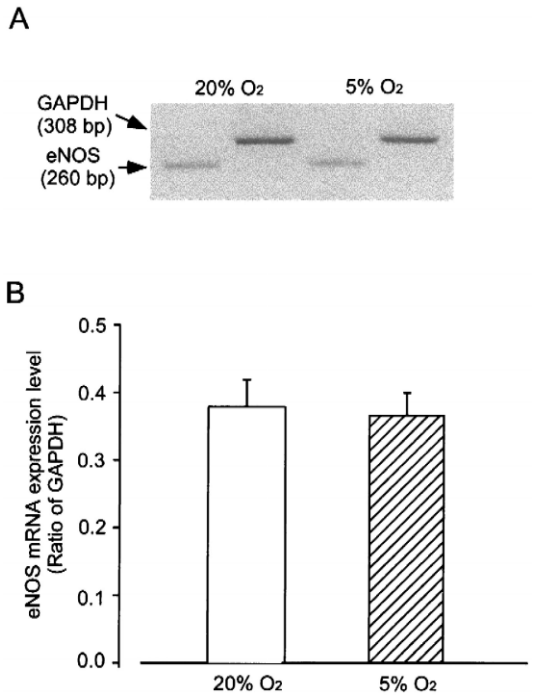

Fig. 4. Semiquantitative RT-PCR for determination of eNOS mRNA in endothelium-intact pulmonary arteries cultured under normoxic (20% O_2) and hypoxic (5% O_2) conditions for 7 days. Agarose-gel electrophoresis of RT-PCR products for eNOS and GAPDH after 30 cycles of amplification. Total RNA was isolated from endothelium-intact normoxic or hypoxic pulmonary arteries. Agarose-gel electrophoresis demonstrated RT-PCR products of expected size corresponding to mRNA encoding eNOS (260 base pairs) and GAPDH (308 base pairs) (A: typical trace of agarose-gel electrophoresis of RT-PCR products; B: quantitative graph showing eNOS mRNA expression level). Results are expressed as means $\pm$ S.E.M. ($n = 5$ each).

cGMP content maximally. In the normoxic pulmonary arteries, the 2-min treatment with substance P (10 nM) increased the cGMP content to 130.5 ± 4.5 pmol/mg ($n = 5$), which was significantly greater than that in hypoxic pulmonary arteries (68.2 ± 3.1; $n = 5$; $P < 0.01$).

3.5. Expression of eNOS mRNA

We examined the effect of hypoxia on the level of mRNA for eNOS using semiquantitative RT-PCR. Amplification at 27–39 cycles showed a step-wise and similar increase in mRNA signals for eNOS (260 base pairs) and GAPDH (308 base pairs) in the normoxic and hypoxic arteries (data not shown). Fig. 4A shows typical results of agarose-gel electrophoresis of the expression of RT-PCR product encoding eNOS and GAPDH after 30 cycles of amplification. Fig. 4B summarizes these results, showing that eNOS mRNA expression relative to GAPDH mRNA expression in the hypoxic arteries did not differ from that in the normoxic arteries at 30 cycles of amplification (0.37 ± 0.03 in the hypoxic pulmonary arteries and 0.38 ± 0.04 in the normoxic pulmonary arteries; $n = 5$, each).

3.6. Expression of eNOS and caveolin-1 protein and measurement of cell area

Fig. 5 shows the pattern of anti-eNOS or anti-caveolin-1 antibody staining in the endothelium of normoxic or hypoxic pulmonary arteries in situ. In order to quantify the amounts of these proteins, the intensity of fluorescence per unit area (1.96×10^4 μm^2) was calculated. The amounts of eNOS and caveolin-1 proteins in hypoxic pulmonary arteries did not differ from those in normoxic pulmonary arteries (eNOS: 180.6 ± 4.0 arbitrary units in hypoxic

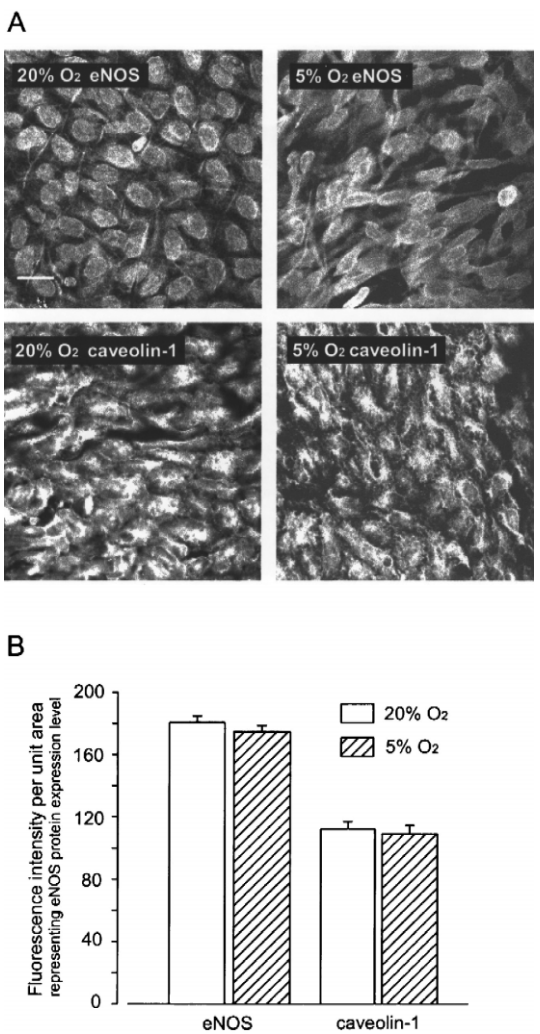

Fig. 5. Whole-mount immunostaining for determination of eNOS and caveolin-1 protein expression in pulmonary arteries cultured under normoxic (20% O_2) or hypoxic (5% O_2) conditions for 7 days. The images were captured with a Carl Zeiss confocal laser scanning microscope LSM510 imaging system. To compare the amount of protein expression in situ, 15-μm-thick images from the endothelial cell surface were digitized under constant exposure time, gain, and offset (A: vascular endothelium stained with anti-eNOS monoclonal antibody and anti-caveolin-1 monoclonal antibody; B: intensity of each pixel in 1.96×10^4 μm^2 of endothelium was measured). Results are expressed as means $\pm$ S.E.M. ($n = 10$ each). Bar = 20 μm.

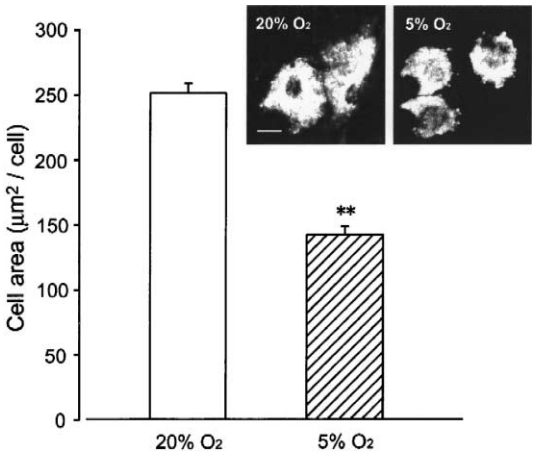

Fig. 6. Whole-mount staining for determination of endothelial cell area in pulmonary arteries cultured under normoxic (20% O_2) or hypoxic (5% O_2) conditions for 7 days. Endothelial cells were stained with a specific endothelial cell marker, Dil-Ac-LDL. The images were captured with a Carl Zeiss confocal laser scanning microscope LSM510 imaging system. To compare the cell area in situ, 5.2-μm-thick sections showing the widest area in each cell were captured under constant exposure time, gain, and offset. The cell area in each cell (including the nucleus) was measured. Results are expressed as means ± S.E.M. ($n = 20$ each). **: significantly different from the normoxic arteries with $P < 0.01$ (inset shows the endothelial cells stained with Dil-Ac-LDL). Bar = 5 μm.

arteries and 171.7 ± 4.0 arbitrary units in normoxic arteries; caveolin-1 112.3 ± 4.9 arbitrary units in hypoxic arteries and 108.8 ± 5.0 arbitrary units in normoxic arteries; $n = 10$ each).

At increased magnification, the distribution of these proteins showed that the endothelial cells were blocked up like cobblestones in the normoxic arteries and that the distribution of these two proteins was equal. However, in the hypoxic arteries, many endothelial cells were shriveled, and eNOS and caveolin-1 proteins were condensed on the periphery of the cell membrane.

To further confirm the atrophy of endothelial cells under hypoxic conditions, we stained the endothelial cells with a specific endothelial cell marker, Dil-Ac-LDL (Fig. 6). We also compared the cell area in the normoxic and hypoxic arteries by calculating the intensity of Dil-Ac-LDL fluorescence. The cell area in hypoxic pulmonary arteries (144.5 ± 6.5 μm^2/cell; $n = 20$) was significantly smaller than that in normoxic pulmonary arteries (254.3 ± 10.5 μm^2/cell; $n = 20$; $P < 0.01$).

4. Discussion

In preliminary experiments, we examined the effects of serum-free organ culture on vascular morphology, contractility, and relaxation. We also confirmed that the vascular morphology was well maintained and that smooth muscle contractility and endothelium-dependent relaxation were preserved in rabbit pulmonary and mesenteric arteries cultured in serum-free DMEM for 7 days (data not shown).

To examine the vasorelaxation by substance P, we attempted to use noradrenaline- or endothelin-1-induced precontraction in hypoxic pulmonary arteries. However, these two agonists did not induce stable tonic contraction in the pulmonary arteries (data not shown). On the contrary, prostaglandin F_{2a} did not induce stable contraction in cultured mesenteric arteries (data not shown). Therefore, we chose prostaglandin F_{2a} and high K^+ as precontractor in pulmonary arteries and noradrenaline and high K^+ in mesenteric arteries. In the present study, chronic hypoxia impaired endothelium-dependent relaxation induced by either substance P or ionomycin in pulmonary arteries (Fig. 1). In contrast, chronic hypoxia did not affect the endothelium-dependent relaxation in mesenteric arteries (Fig. 2). These results suggest that the impairment of endothelium-dependent relaxation by chronic hypoxia is specifically observed in pulmonary arteries. The impairment of the endothelium-dependent relaxation observed in the organ culture study is also consistent with the impairment of endothelium-dependent relaxation in pulmonary arteries from pulmonary hypertensive rats and with the impairment seen in patients with pulmonary hypertension (Xue and Johns, 1995; Giaid and Saleh, 1995; Shaul et al., 1993; Nakazawa et al., 1999). These results suggest that vessels cultured under conditions of chronic hypoxia form a useful in vitro model for pulmonary hypertension. In this study, we examined the mechanisms of the hypoxia-induced impairment of endothelium-dependent relaxation using this model.

In the first step, we examined the effects of chronic hypoxia on the soluble guanylate cyclase/cGMP/G kinase pathway in smooth muscle cells. As shown in Fig. 3B, the sodium nitroprusside-induced increase in cGMP content did not change in the pulmonary arteries treated with hypoxia for 7 days. Additionally, the responsiveness of smooth muscle cells to sodium nitroprusside was not changed by chronic hypoxia (Fig. 3A). These results suggest that chronic hypoxia does not affect the guanylate cyclase/cGMP/G kinase pathway in smooth muscle cells, and that the impairment of endothelium-dependent relaxation may be attributable to endothelial dysfunction.

Next, the change in the amount of eNOS mRNA was examined by semiquantitative RT-PCR analysis. In the present experiments, we performed the hot start method using Ampli Taq Gold DNA polymerase (Roche). Ampli Taq Gold requires more PCR cycle number to obtain sufficient product yielded than conventional recombinant Taq. Indeed, we obtained plateau densitometric intensity of GAPDH at 36 PCR cycles (data not shown). We confirmed step-wise increase in PCR products, and employed the results at 30 PCR cycles for quantitative determination. The amount of eNOS protein was also determined by measuring the intensity of fluorescence per unit area in whole-mount immunostained tissue. The results showed

that eNOS mRNA and protein expression in the endothelium were not affected by chronic hypoxia (Figs. 4 and 5), suggesting that the impairment of endothelium-dependent relaxation may not be due to a decrease in eNOS expression. Others have reported that eNOS mRNA and/or protein expression and enzyme activity were increased in pulmonary vessels and lung homogenates prepared from hypoxia- and monocrotaline-induced pulmonary hypertensive rats (Xue and Johns, 1996; Nakazawa et al., 1999; Resta et al., 1997; Le Cras et al., 1996). It has also been reported that shear stress, neurohumoral stimuli such as various growth factors, and hemodynamics stimulate eNOS mRNA expression (Sessa et al., 1994; Xiao et al., 1997; Förstermann et al., 1995). In the animal models mentioned above, these stimulatory effects may be involved in the up-regulation of eNOS.

The results of the present study also showed that the increase in cGMP content by substance P was significantly decreased in the arteries treated with chronic hypoxia (Fig. 3B). In addition, endothelium-dependent relaxation induced not only by receptor stimulation but also by the Ca^{2+} ionophore, ionomycin, was impaired (Fig. 1B and C). These results suggest the possibility that a functional disturbance of eNOS might occur in pulmonary arterial endothelial cells after chronic hypoxia.

It has been reported that depletion of a NO precursor, L-arginine, and of a cofactor, tetrahydrobiopterin, leads to reduced NO production in the vascular endothelium (Eddahibi et al., 1992; Maier et al., 2000). Furthermore, it is possible that endogenous superoxide anions scavenge the produced NO (Rubanyi and Vanhoutte, 1986; Beckman et al., 1990). In the present study, however, we found that the addition of L-arginine, tetrahydrobiopterin or superoxide dismutase did not restore the impaired endothelium-dependent relaxation. Thus, it might be that neither depletion of a substrate or cofactor nor elimination of NO by superoxide was involved in the chronic hypoxia-induced impairment of endothelium-dependent relaxation.

eNOS is dynamically targeted to specialized cell-surface signal-transducing domains termed plasmalemmal caveolae, and the localization of eNOS in caveolae is required for the efficient and controlled production of NO. Caveolin-1, an integral membrane protein that comprises a key structural component of caveolae, is known to interact with eNOS (Garcia-Cardena et al., 1997; Liu et al., 1996). In endothelial cells, the inhibitory eNOS-caveolin-1 complex is disrupted by binding of Ca^{2+}-calmodulin to eNOS, leading to enzyme activation (Michel et al., 1997; Garcia-Cardena et al., 1997) and an enhanced expression and interaction of caveolin-1 with eNOS contribute to an impairment of NO production and reduced eNOS activity (Shah et al., 1999; Hare et al., 2000). In the present study, it was found that the expression of caveolin-1 protein in the endothelium did not change after chronic hypoxia (Fig. 5). Our morphological study clearly showed atrophy of the endothelium after chronic hypoxia (Figs. 5A and 6). In previous reports, a coarse endothelial surface was observed in pulmonary arteries from patients and rats with pulmonary hypertension (Rabinovitch et al., 1979; Meyrick and Reid, 1980). Those authors suggested that these morphological changes may have resulted from high pressure or flow. In our study, however, the endothelial cells were shriveled giving rise to an area without endothelial cells in the surface of the inner vascular wall after culture under hypoxic conditions. In addition, condensation of eNOS in the endothelial cells was often observed. These data suggested that a low oxygen tension changed the structure of endothelial cells. These morphological changes in endothelial cells may affect the interaction between eNOS and the cell membrane, and may also result in eNOS enzyme inactivity in situ. Additionally, these morphological changes indicate the possibility that NO produced in endothelial cells might not diffuse efficiently into the smooth muscle cell layer.

The mechanism for the selective impairment of endothelium-dependent relaxation in the pulmonary artery was not clarified in the present experiments. Recently, various genes including hypoxia-inducible factor-1α (HIF-1α) and HIF-1α-like factor (HLF) were reported to be involved in the pathogenesis of pulmonary hypertension due to chronic hypoxia, and the expression of these genes was different among tissues (Iyer et al., 1998; Ryan et al., 1998; Tian et al., 1998). Therefore, tissue differences in gene expression may be related to the selective impairment in the pulmonary artery. Further investigations are needed to clarify this possibility.

In summary, organ culture of pulmonary arteries under conditions of chronic hypoxia is a useful in vitro model for pulmonary hypertension. Chronic hypoxia impaired NO-mediated endothelium-dependent relaxation without changing the amount of eNOS protein or mRNA expression, and without changing the NO sensitivity of smooth muscle cells. The dysfunction of the endothelium-dependent relaxation may possibly be explained by morphological changes in endothelial cells caused by chronic hypoxia resulting in the dissociation of eNOS and cell-membrane structural components, such as caveolin-1.

Acknowledgements

This work was partly supported by a Grant-in-Aid for scientific research from the Ministry of Education, Japan.

References

Arnet, U.A., McMillan, A., Dinerman, J.L., Ballermann, B., Lowenstein, C.J., 1996. Regulation of endothelial nitric-oxide synthase during hypoxia. J. Biol. Chem. 271, 15069–15073.

Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A., Freeman, B.A., 1990. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc. Natl. Acad. Sci. U. S. A. 87, 1620–1624.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Eddahibi, S., Adnot, S., Carville, C., Blouquit, Y., Raffestin, B., 1992. L-arginine restores endothelium-dependent relaxation in pulmonary circulation of chronically hypoxic rats. Am. J. Physiol. 263, L194–L200.

Förstermann, U., Gath, I., Schwarz, P., Closs, E.I., Kleinert, H., 1995. Isoforms of nitric oxide synthase. Properties, cellular distribution and expressional control. Biochem. Pharmacol. 50, 1321–1332.

Fulton, D., Gratton, J.P., McCabe, T.J., Fontana, J., Fujio, Y., Walsh, K., Franke, T.F., Papapetropoulos, A., Sessa, W.C., 1999. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature 399, 597–601.

Garcia-Cardena, G., Martasek, P., Masters, B.S., Skidd, P.M., Couet, J., Li, S., Lisanti, M.P., Sessa, W.C., 1997. Dissecting the interaction between nitric oxide synthase (NOS) and caveolin. Functional significance of the nos caveolin binding domain in vivo. J. Biol. Chem. 272, 25437–25440.

Giaid, A., Saleh, D., 1995. Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. N. Engl. J. Med. 333, 214–221.

Griendling, K.K., Alexander, R.W., 1996. Endothelial control of the cardiovascular system: recent advances. FASEB J. 10, 283–292.

Gruetter, C.A., Gruetter, D.Y., Lyon, J.E., Kadowitz, P.J., Ignarro, L.J., 1981. Relationship between cyclic guanosine 3′:5′-monophosphate formation and relaxation of coronary arterial smooth muscle by glyceryl trinitrate, nitroprusside, nitrite and nitric oxide: effects of methylene blue and methemoglobin. J. Pharmacol. Exp. Ther. 219, 181–186.

Hare, J.M., Lofthouse, R.A., Juang, G.J., Colman, L., Ricker, K.M., Kim, B., Senzaki, H., Cao, S., Tunin, R.S., Kass, D.A., 2000. Contribution of caveolin protein abundance to augmented nitric oxide signaling in conscious dogs with pacing-induced heart failure. Circ. Res. 86, 1085–1092.

Iyer, N.V., Kotch, L.E., Agani, F., Leung, S.W., Laughner, E., Wenger, R.H., Gassmann, M., Gearhart, J.D., Lawler, A.M., Yu, A.Y., Semenza, G.L., 1998. Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev. 12, 149–162.

Le Cras, T.D., Xue, C., Rengasamy, A., Johns, R.A., 1996. Chronic hypoxia upregulates endothelial and inducible NO synthase gene and protein expression in rat lung. Am. J. Physiol. 270, L164–L170.

Liao, J.K., Zulueta, J.J., Yu, F.S., Peng, H.B., Cote, C.G., Hassoun, P.M., 1995. Regulation of bovine endothelial constitutive nitric oxide synthase by oxygen. J. Clin. Invest. 96, 2661–2666.

Liu, J., Garcia-Cardena, G., Sessa, W.C., 1996. Palmitoylation of endothelial nitric oxide synthase is necessary for optimal stimulated release of nitric oxide: implications for caveolae localization. Biochemistry 35, 13277–13281.

Maier, W., Cosentino, F., Lutolf, R.B., Fleisch, M., Seiler, C., Hess, O.M., Meier, B., Luscher, T.F., 2000. Tetrahydrobiopterin improves endothelial function in patients with coronary artery disease. J. Cardiovasc. Pharmacol. 35, 173–178.

McQuillan, L.P., Leung, G.K., Marsden, P.A., Kostyk, S.K., Kourembanas, S., 1994. Hypoxia inhibits expression of eNOS via transcriptional and posttranscriptional mechanisms. Am. J. Physiol. 267, H1921–H1927.

Meyrick, B., Reid, L., 1980. Endothelial and subintimal changes in rat hilar pulmonary artery during recovery from hypoxia. A quantitative ultrastructural study. Lab. Invest. 42, 603–615.

Michel, J.B., Feron, O., Sacks, D., Michel, T., 1997. Reciprocal regulation of endothelial nitric-oxide synthase by Ca^{2+}-calmodulin and caveolin. J. Biol. Chem. 272, 15583–15586.

Nakazawa, H., Hori, M., Ozaki, H., Karaki, H., 1999. Mechanisms underlying the impairment of endothelium-dependent relaxation in the pulmonary artery of monocrotaline-induced pulmonary hypertensive rats. Br. J. Pharmacol. 128, 1098–1104.

Nishida, K., Harrison, D.G., Navas, J.P., Fisher, A.A., Dockery, S.P., Uematsu, M., Nerem, R.M., Alexander, R.W., Murphy, T.J., 1992. Molecular cloning and characterization of the constitutive bovine aortic endothelial cell nitric oxide synthase. J. Clin. Invest. 90, 2092–2096.

Phelan, M.W., Faller, D.V., 1996. Hypoxia decreases constitutive nitric oxide synthase transcript and protein in cultured endothelial cells. J. Cell Physiol. 167, 469–476.

Pollock, J.S., Förstermann, U., Mitchell, J.A., Warner, T.D., Schmidt, H.H., Nakane, M., Murad, F., 1991. Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. Proc. Natl. Acad. Sci. U. S. A. 88, 10480–10484.

Rabinovitch, M., Gamble, W., Nadas, A.S., Miettinen, O.S., Reid, L., 1979. Rat pulmonary circulation after chronic hypoxia: hemodynamic and structural features. Am. J. Physiol. 236, H818–H827.

Resta, T.C., Gonzales, R.J., Dail, W.G., Sanders, T.C., Walker, B.R., 1997. Selective upregulation of arterial endothelial nitric oxide synthase in pulmonary hypertension. Am. J. Physiol. 272, H806–H813.

Rubanyi, G.M., Vanhoutte, P.M., 1986. Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor. Am. J. Physiol. 250, H822–H827.

Ryan, H.E., Lo, J., Johnson, R.S., 1998. HIF-1 alpha is required for solid tumor formation and embryonic vascularization. Embo. J. 17, 3005–3015.

Sessa, W.C., Pritchard, K., Seyedi, N., Wang, J., Hintze, T.H., 1994. Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. Circ. Res. 74, 349–353.

Shah, V., Toruner, M., Haddad, F., Cadelina, G., Papapetropoulos, A., Choo, K., Sessa, W.C., Groszmann, R.J., 1999. Impaired endothelial nitric oxide synthase activity associated with enhanced caveolin binding in experimental cirrhosis in the rat. Gastroenterology 117, 1222–1228.

Shaul, P.W., Wells, L.B., Horning, K.M., 1993. Acute and prolonged hypoxia attenuate endothelial nitric oxide production in rat pulmonary arteries by different mechanisms. J. Cardiovasc. Pharmacol. 22, 819–827.

Tian, H., Hammer, R.E., Matsumoto, A.M., Russell, D.W., McKnight, S.L., 1998. The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. Genes Dev. 12, 3320–3324.

Xiao, Z., Zhang, Z., Ranjan, V., Diamond, S.L., 1997. Shear stress induction of the endothelial nitric oxide synthase gene is calcium-dependent but not calcium-activated. J. Cell Physiol. 171, 205–211.

Xue, C., Johns, R.A., 1995. Endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. N. Engl. J. Med. 333, 1642–1644.

Xue, C., Johns, R.A., 1996. Upregulation of nitric oxide synthase correlates temporally with onset of pulmonary vascular remodeling in the hypoxic rat. Hypertension 28, 743–753.

Yamawaki, H., Sato, K., Hori, M., Ozaki, H., Karaki, H., 1999. Impairment of endothelium-dependent relaxation in the arteries cultured with fetal bovine serum. Eur. J. Pharmacol. 366, 237–242.

Zapol, W.M., Jones, R., 1987. Vascular components of ARDS. Clinical pulmonary hemodynamics and morphology. Am. Rev. Respir. Dis. 136, 471–474.

Zapol, W.M., Kobayashi, K., Snider, M.T., Greene, R., Laver, M.B., 1977. Vascular obstruction causes pulmonary hypertension in severe acute respiratory failure. Chest 71, 306–307.