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Research Article

Oxygen (O2) may regulate pulmonary vascular resistance through changes in endothelial nitric oxide (NO) production. To determine whether constitutive NO synthase (cNOS) is regulated by O2, we assessed cNOS expression and activity in bovine pulmonary artery endothelial cells exposed to different concentrations of O2. In a time-dependent manner, changes in O2 concentration from 95 to 3% produced a progressive decrease in cNOS mRNA and protein levels resulting in 4.8- and 4.3-fold reductions after 24h, respectively. This correlated with changes in cNOS activity as determined by nitrite measurements. Compared with 20% O2, cNOS activity was increased 1.5-fold in 95% O2 and decreased 1.9-fold in 3% O2. A decrease in O2 concentration from 94 to 3% shortened cNOS mRNA half-life from 46 to 24 h and caused a 20-fold repression of cNOS gene transcription. Treatment with cycloheximide produced a threefold increase in cNOS mRNA at all O2 concentrations, suggesting that cNOS mRNA expression is negatively regulated under basal condition. We conclude that O2 upregulates cNOS expression through transcriptional and post-transcriptional mechanisms. A decrease in cNOS activity in the presence of low O2 levels, therefore, may contribute to hypoxia-induced vasoconstriction in the pulmonary circulation.

Regulation of Bovine Endothelial Constitutive Nitric Oxide Synthase by Oxygen

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Abstract

Oxygen (O_2) may regulate pulmonary vascular resistance through changes in endothelial nitric oxide (NO) production. To determine whether constitutive NO synthase (cNOS) is regulated by O_2, we assessed cNOS expression and activity in bovine pulmonary artery endothelial cells exposed to different concentrations of O_2. In a time-dependent manner, changes in O_2 concentration from 95 to 3% produced a progressive decrease in cNOS mRNA and protein levels resulting in 4.8- and 4.3-fold reductions after 24 h, respectively. This correlated with changes in cNOS activity as determined by nitrite measurements. Compared with 20% O_2, cNOS activity was increased 1.5-fold in 95% O_2 and decreased 1.9-fold in 3% O_2. A decrease in O_2 concentration from 95 to 3% shortened cNOS mRNA half-life from 46 to 24 h and caused a 20-fold repression of cNOS gene transcription. Treatment with cycloheximide produced a threefold increase in cNOS mRNA at all O_2 concentrations, suggesting that cNOS mRNA expression is negatively regulated under basal condition. We conclude that O_2 upregulates cNOS expression through transcriptional and post-transcriptional mechanisms. A decrease in cNOS activity in the presence of low O_2 levels, therefore, may contribute to hypoxia-induced vasoconstriction in the pulmonary circulation. (*J. Clin. Invest.* 1995. 96:2661–2666.) Key words: pulmonary • hypoxia • hyperoxia • gene regulation

Introduction

The pulmonary arterial vasculature responds to changes in oxygen tension hemodynamically and structurally (1). Although acute hypoxia raises pulmonary arterial pressure and resistance (2), the factor (or factors) responsible for this vasomotor response, which may involve the excess release of a vasoconstrictor such as endothelin or impaired release of a vasodilator such as the endothelium-derived relaxing factor (EDRF),[1] has (have) not been clearly identified (3). There is now ample evidence for endothelium-dependent relaxation of vascular smooth muscle cells in pulmonary vessels in response to known stimulants of EDRF, such as acetylcholine or bradykinin, both in humans (4, 5) and in animals (6, 7). In addition, several studies have suggested that the production or activity of EDRF, now identified as nitric oxide (NO) or a related compound, may mediate oxygen-induced changes in pulmonary vascular resistance (8–12). The source of NO production can be assumed to be the vascular endothelium because of known receptor-mediated release of NO from this cellular source (13) and recent data demonstrating the presence of NO synthase (NOS) in human and rat lung endothelium (14). Although the endothelium can express two forms of NOS, NO is largely derived from constitutively expressed endothelial NOS (cNOS) under most physiologic conditions. The inducible isoform of NOS is usually not present under basal conditions but can be transcriptionally induced during inflammation by cytokines (15). Thus, this study was undertaken to determine whether oxygen can regulate cNOS expression and activity in bovine pulmonary artery endothelial cells (EC).

Methods

Reagents. All standard culture reagents were obtained from Gibco Laboratories (Grand Island, NY). FCS was obtained from Hyclone Laboratories, Sterile System (Logan, UT). Penicillin G potassium was from Squibb Pharmaceuticals (Princeton, NJ). Streptomycin sulfate was from Pfizer Company (New York). Actinomycin D, cycloheximide, PMSF, leupeptin, aprotinin, DTT, *N*-nitro-L-arginine methyl ester hydrochloride (L-NAME), nitrate reductase, and BSA were purchased from Sigma Chemical Co. (St. Louis, MO). [α-^{32}P]CTP (3,000 Ci/mmol) and [α-^{32}P]UTP (800 Ci/mmol) were supplied by New England Nuclear (Boston, MA). Protein molecular weight markers were purchased from Bethesda Research Laboratories, Inc. (Bethesda, MD). The Western blotting kit (enhanced chemiluminescence) using horseradish peroxidase and Luminol was obtained from Amersham Corp. (Arlington Heights, IL). Nylon transfer membranes were purchased from Schleicher and Schuell (Dassel, Germany).

Isolation and culture of cells. Bovine pulmonary artery and aortic EC were isolated, characterized, and subcultured according to previously described methods (16). Second- to fifth-passage cells were used for all experiments and were cultured in a growth medium containing RPMI 1640, 5% FCS, and antibiotic mixture of 100 U/ml penicillin/100 μg/ml streptomycin/250 ng/ml Fungizone. In some experiments, cells were pretreated with either actinomycin D (1 μg/ml) or cycloheximide (1 μg/ml) for 1 h before exposure to changes in O_2 concentration. Cellular confluence was maintained for all treatment conditions. Cellular viability was determined by Trypan blue exclusion and ^{51}Cr release assay as described previously (16).

Exposure of cells to oxygen concentration. Confluent bovine pulmonary artery EC were placed in humidified airtight incubation chambers (Billups-Rothenberg, Del Mar, CA) and gassed with various concentrations of oxygen (0, 3, 10, 20, and 95% O_2), 5% CO_2, and balance N_2 for various periods of time (0–72 h). The chambers were maintained in an incubator (New Brunswick Scientific, Edison, NJ) for the duration

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1. *Abbreviations used in this paper:* cNOS, constitutive NO synthase; EC, endothelial cells; EDRF, endothelium-derived relaxing factor; L-NAME, *N*-nitro-L-arginine methyl ester hydrochloride; NO, nitric oxide; NOS, NO synthase.

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of exposure. The percentages of O_2 in the chambers were routinely checked (LB-2 O_2 analyzer; Beckman Instruments, Inc., Fullerton, CA) at 24 and 48 h of exposure and were consistently within 2% of the desired O_2 concentration. The percentages of CO_2 were also monitored (OM-11 CO_2 analyzer; Beckman Instruments, Inc.) and were within 4–6% for all oxygen exposures.

Assessment of cell integrity. Cell integrity was routinely assessed by morphological examination of the cells under phase-contrast microscopy, trypan blue exclusion, and ^{51}Cr release assay as described previously (16).

Northern blotting. Equal amounts of total RNA (20 μg/lane) were separated by 1.2% formaldehyde-agarose gel electrophoresis, transferred overnight onto nitrocellulose membrane by capillary action, and baked (72°C) for 2 h before hybridization. The full-length human endothelial cNOS cDNA (17) was labeled using random hexamer priming with [α-^{32}P]CTP and Klenow fragment of DNA polymerase I (Pharmacia LKB Biotechnology, Piscataway, NJ) and hybridized to membranes overnight at 45°C in a solution containing 50% formamide, 5× SSC, 2.5× Denhardt's solution, 25 mM sodium phosphate buffer (pH 6.5), 0.1% SDS, and 250 μg/ml salmon sperm DNA (Sigma Chemical Co.). All Northern blots were subjected to stringent washing conditions (0.2× SSC/0.1% SDS at 68°C) before autoradiography with intensifying screen at −80°C. Loading conditions were determined by subsequent rehybridization of blots with β-tubulin cDNA. In some studies, blots were hybridized to human smooth muscle or murine macrophage inducible NOS cDNA probe.

In vitro transcription studies. Nuclei from EC (4×10^7 cells) exposed to the indicated O_2 concentration from 12 h were isolated as described previously (18). In vitro transcription was carried out in a shaking water bath at 30°C for 30 min in a buffer containing 10 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 300 mM KCl, 50 μM EDTA, 1 mM DTT, 0.5 U RNAsin, 0.5 mM CTP, ATP, GTP, and 250 μCi [α-^{32}P]-UTP. The reaction was terminated by incubating the assay with 40 U of DNase I (Ambion Inc., Austin, TX) for 20 min at 30°C. Proteins in the mixture were degraded by a solution containing 0.4% SDS, 40 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 400 μg/ml of proteinase K (E. Merck, Darmstadt, Germany), and extracted by phenol/chloroform. Radiolabeled RNA transcripts were isolated by cesium chloride ultracentrifugation.

Equal amounts (1 μg) of linearized, denatured cDNA corresponding to NOS, pGEM, and β-tubulin were vacuum-transferred onto nylon membranes using a slot blot apparatus (Schleicher and Schuell). The membranes were baked and prehybridized as described for Northern blots. Radiolabeled transcripts ($\sim 5 \times 10^7$ cpm) were resuspended in 2 ml of hybridization buffer containing 50% formamide, 5× SSC, 2.5× Denhardt's Solution, 25 mM sodium phosphate buffer (pH 6.5), 0.1% SDS, and 250 μg/ml salmon sperm DNA. Hybridization of radiolabeled transcripts to the nylon membranes was carried out at 45°C for 48 h. The membranes were then washed with 1× SSC/0.1% SDS for 1 h at 65°C before autoradiography for 72 h at −80°C.

Western blotting. Partially purified membranes were prepared as described previously (19). Protein concentration was determined by the method of Lowry (20). Membranes proteins (50 μg) and molecular weight markers were separated by SDS/PAGE (12% running, 4% stacking gel). The proteins were electrophoretically transferred onto nylon membrane (PVDF, Schleicher and Schuell) and incubated overnight at 4°C with blocking solution (5% dried skim milk in PBS) before the addition of goat anti-cNOS antisera (1:400 dilution) or goat anti-inducible NOS antisera (1:600). The blots were washed twice with PBS buffer containing 0.1% Tween 20 and then treated with rabbit anti-goat horseradish peroxidase antibody (1:8,000 dilution; Pierce, Rockford, IL). Immunodetection was accomplished using an enhanced chemiluminescence kit (Amersham Corp.).

Measurement of nitrite production. The release of nitrite by EC in culture medium was determined using a method described by Misko et al. (21), with minor modifications. This is based on the conversion, in the presence of nitrite and in acidic condition, of nonfluorescent 2,3-diaminonaphtalene (DAN) to the fluorescent compound 1-(H)-naphtotriazole (NT). Briefly, EC monolayers were exposed to various concentrations of O_2 for 24 h. After exposure, the medium was removed, and the cells were washed and incubated for an additional 6 h in fresh phenol red–free medium with or without L-NAME (30 μM). At the end of incubation, 850 μl of medium was removed, treated with nitrate reductase to convert nitrate into nitrite, and then mixed with 100 μl DAN (0.05 mg/ml in 0.62 M HCl). The mixture was protected from light and incubated for 10 min at 20°C, after which the reaction was terminated by adding 50 μl of 2.8 N NaOH. Fluorescence of NT was measured using excitation and emission wavelengths of 365 and 450 nm, respectively. Quantification of nitrite was assessed using standard curves obtained with pure nitrite and prepared with each experiment. NO production was estimated as the L-NAME–inhibitable portion of the total nitrite production of the cells over 6 h.

Data analysis. Band intensities from Northern, Western, and nuclear run-off assay blots were analyzed densitometrically by the National Institutes of Health Image program (22). All values are expressed as mean±SEM compared with controls and among separate experiments. Paired and unpaired Student's *t* tests were used to determine any significant changes in densitometric values. A significant difference was taken for P values < 0.05.

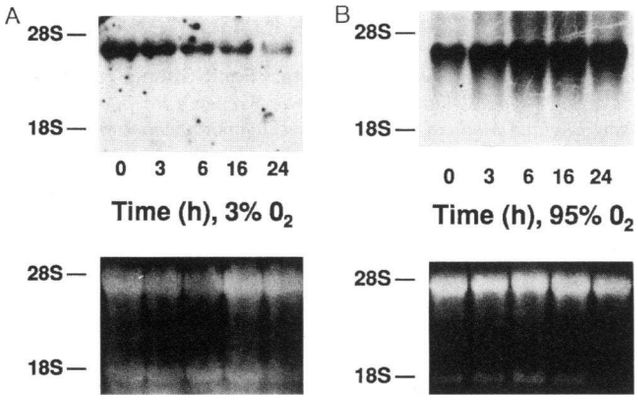

Figure 1. Northern blots (20 μg total RNA/lane) showing the time-dependent effects of (*A*) hypoxia (3% O_2) and (*B*) hyperoxia (95% O_2) on cNOS steady state mRNA levels. RNA loading was assessed by ethidium bromide staining of the gel. This is representative of three separate experiments.

Results

Cell culture. Relatively pure (> 95%) bovine pulmonary artery EC cultures were confirmed by their morphological features (i.e., cuboidal, cobblestone, contact inhibited) using phase-contrast microscopy and by immunofluorescent staining with Factor VIII antibodies. There were no observable adverse effects of oxygen tension, actinomycin D, and cycloheximide on cellular viability and morphology for all treatment conditions.

Effect of oxygen tension on EC cNOS mRNA expression. As shown in Fig. 1 *A*, exposure of EC to hypoxia (3% O_2) caused a progressive decrease in cNOS steady state mRNA levels in a time-dependent manner. After 24 h of exposure, cNOS mRNA level was reduced 2.7-fold compared with baseline (time 0, 20% O_2). Conversely, exposure of EC to hyperoxia (95% O_2) produced a progressive increase in cNOS mRNA level resulting in a 1.8-fold increase at 24 h of exposure, as compared with baseline (Fig. 1 *B*). In an oxygen tension–dependent manner, a decrease from 95 to 0% O_2 caused a 12.9-fold reduction in steady state cNOS mRNA level after 24 h of

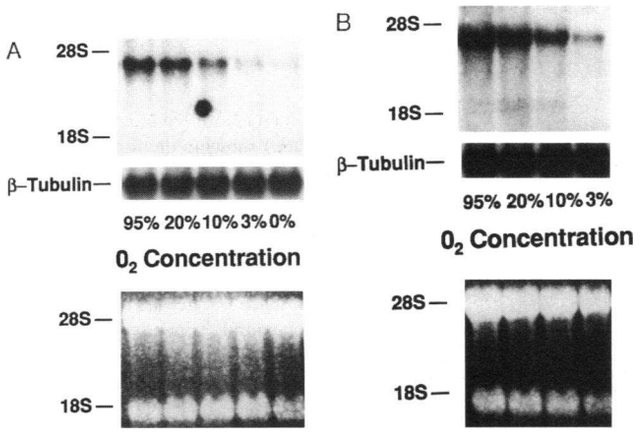

Figure 2. Northern blots (20 μg total RNA/lane) showing the effects of various concentrations of oxygen on bovine pulmonary artery (*A*) and aortic (*B*) EC cNOS mRNA levels after 24 h of exposure. RNA loading was determined by hybridization to β-tubulin and ethidium bromide staining of the gel. This is representative of two separate experiments.

exposure (Fig. 2 *A*). Cell counts were not significantly affected by the various O_2 exposures (2.1±0.03, 2.2±0.01, 2.6±0.03, 2.6±0.03, and 2.5±0.03 million cells, for 0, 3, 10, 20, and 95% O_2, respectively). Similar Northern analyses did not show the presence of inducible NOS on RNA at any level of O_2 tension (data not shown). For comparison, bovine aortic EC were also exposed to various O_2 concentrations. A decrease from 95 to 3% O_2 caused a 14.6-fold reduction in steady state cNOS mRNA level after 24 h of exposure (Fig. 2 *B*). Cell counts were comparable under various O_2 exposures (1.6±0.03, 1.4±0.03, 1.5±0.07, and 1.6±0.05 million cells, for 3, 10, 20, and 95% O_2, respectively).

In the presence of actinomycin D, the posttranscriptional half-life of cNOS mRNA was reduced by exposure to decreasing oxygen tension (Fig. 3 *A*). The calculated half-lives of cNOS mRNA were 46±5, 34±5, and 24±4 h in 95, 20, and 3% O_2, respectively ($P < 0.05$) (Fig. 3 *B*). These findings indicate that the stability of cNOS mRNA is, in part, regulated by oxygen tension. The protein synthesis inhibitor, cycloheximide, however, caused a time-dependent increase in steady state cNOS mRNA level from EC exposed to 95, 20, and 3% O_2 compared with their counterparts, untreated control cells exposed to the same O_2 tension (Fig. 4 *A*). After 24 h, there was approximately a threefold increase in cNOS mRNA in the presence of cycloheximide compared with controls at all O_2 concentrations (Fig. 4, *A* and *B*). These results raise the possibility that continuous protein synthesis may be required for cNOS mRNA destabilization and suggest that cNOS mRNA expression is negatively regulated under basal condition. The decrease in cNOS mRNA expression observed in EC treated with cycloheximide at all three O_2 concentrations after 48 h of exposure is most likely a toxic effect related to overall inhibition of protein synthesis after such prolonged exposure.

Effect of oxygen tension on cNOS gene transcription. Nuclear run-on studies demonstrated that transcriptional activity of the cNOS gene was dependent upon oxygen tension with respect to β-tubulin gene transcription (Fig. 5). Exposure of EC to 95% O_2 produced a 1.8-fold increase in transcriptional activity of cNOS compared with EC exposed to 20% O_2 (control cells). Similarly, exposure of EC to 3% O_2 resulted in an 11.5-fold decrease in cNOS gene transcription compared with control cells. The presence of cycloheximide, however, did not affect cNOS gene transcription (data not shown). Preliminary studies using different amounts of radiolabeled RNA transcripts demonstrate that, under our experimental conditions, hybridization was linear and nonsaturable. The specificity of each band was determined by the lack of hybridization to the insertless pGEM vector cDNA.

Effects of oxygen tension on cNOS protein expression. The protein level of cNOS was also affected by changes in oxygen tension. Compared with control EC exposed to 20% O_2, exposure of EC to 95% O_2 produced a 1.8-fold increase in cNOS protein levels after 24 h, while exposure of EC to 3 and 0% O_2 produced 2.4- and 3.1-fold decreases, respectively (Fig. 6). Exposure to hyperoxia (95% O_2) caused a small time-dependent increase in cNOS protein levels resulting in a 1.5-fold increase after 72 h (Fig. 7 *A*). Exposure of EC to hypoxia (3% O_2), however, produced 2.5- and 4.2-fold decreases in cNOS protein levels at 24 and 72 h, respectively (Fig. 7 *B*). There were no changes in cNOS protein levels under normoxic conditions (20% O_2) (data not shown).

Effect of oxygen tension on NOS activity. Nitrite production from EC that had been previously exposed to 3, 20, and 95% O_2 for 12 h was measured as detailed in Methods. The L-NAME–inhibitable portion of this production was estimated to be derived from NO. As shown in Fig. 8, the L-NAME–inhibitable production of nitrite by EC was significantly reduced in

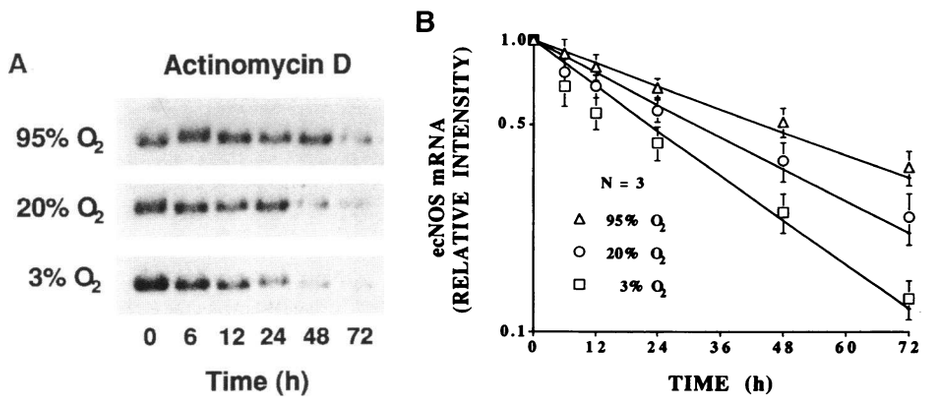

Figure 3. (*A*) Northern blots (20 μg total RNA/lane) showing the time course of cNOS mRNA expression in the presence of actinomycin D (1 μg/ml) and various oxygen concentrations. (*B*) Graphic depiction of the effects of actinomycin D and oxygen concentration on cNOS mRNA levels. Band intensities were analyzed by densitometry, normalized to β-tubulin mRNA, and plotted logarithmically as a function of time (relative intensity). Studies were performed three times.

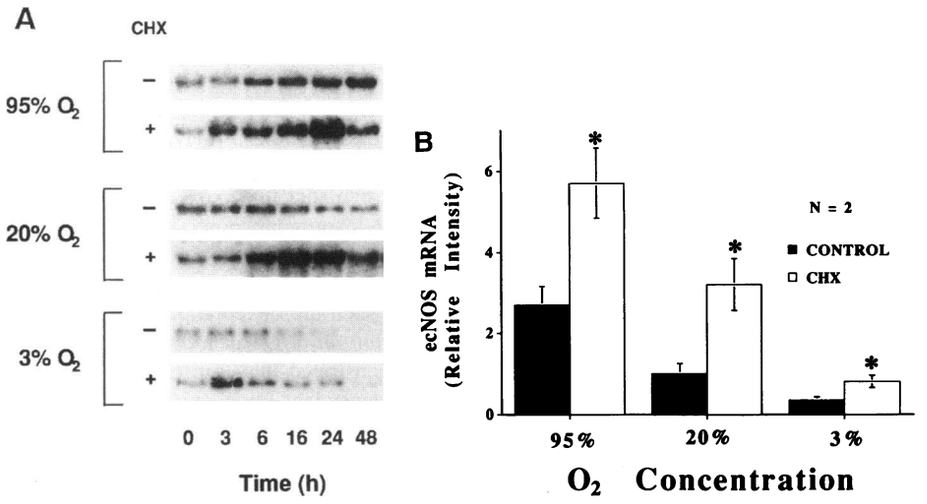

Figure 4. (*A*) Northern blots (20 μg total RNA/lane) showing the time course changes of cNOS mRNA in the presence of cycloheximide (*CHX*, 1 μg/ml) and under various oxygen concentrations. (*B*) Graphic depiction of the effects of oxygen concentration on cNOS mRNA levels in the absence (*filled bars*) and presence of cycloheximide (*open bars*, 1 μg/ml) at 24 h. Band intensities were analyzed by densitometry and normalized to β-tubulin mRNA (relative intensity). Each study was performed twice in duplicate. Asterisks indicate $P < 0.05$ vs. control for each O_2 exposure.

hypoxia (3% O_2, 64±17 nmol/10^6/6 h) and increased in hyperoxia (95% O_2, 186±25 nmol/10^6/6 h), as compared with normoxia (20% O_2, 123±15 nmol/10^6/6 h).

Discussion

The specific response of the EC to changes in O_2 tension in relation to synthesis and release of NO is not known. Conflicting conclusions have been drawn from experiments performed either in intact animals or isolated vessels. For example, depending on the study, hypoxia has been shown to either stimulate (23) or inhibit (24) the release of EDRF from isolated vascular segments. That NOS requires molecular O_2 but that NO stability may be increased in hypoxia, due to decreased reactive O_2 species such as superoxide which normally inactivates NO (25), are confounding factors in these experiments. Possible O_2-related changes in function or availability of substrates or cofactors of NOS add another degree of complexity to the interpretation of the results. In a recent study by Shaul and Wells (26) where NO was assayed by measuring cGMP accumulation in fetal vascular smooth muscle cells during coculture incubations of these cells with EC, oxygen was shown to acutely modulate NO production with decreased production at lower O_2 tension. It has been suggested that hyperbaric oxygenation, on the other hand, dilates fetal pulmonary circulation through release of EDRF and that this phenomenon may contribute to the postnatal adaptation of the pulmonary circulation at birth (10).

This study was conducted to investigate the effect of O_2 concentration on cNOS expression and activity in cultured pulmonary artery EC. Our results indicate that cNOS gene and protein expression is directly regulated by the O_2 concentration to which the cells are exposed, with a time-dependent increase and decrease in this expression in hyperoxia and hypoxia, respectively. As indicated by nuclear run-on studies and the evaluation of the half-life of cNOS mRNA in the presence of actinomycin D, O_2 tension affects cNOS expression at the levels of both gene transcription and mRNA stability. This effect is more significant at the lower spectrum of O_2 tension considering the larger variation in cNOS gene transcription (11.5-fold decrease versus 1.8-fold increase in hyperoxia) produced by hypoxia for a relatively smaller change in O_2 concentration as compared with hyperoxia. Therefore, regulation of EC cNOS by O_2 may be more relevant in conditions of hypoxia.

As they relate to hypoxia, these results are in accordance with the study by McQuillan et al. (27) describing decreased NOS transcriptional rate and message stability in hypoxic human umbilical vein EC. Both studies, however, are in contradiction with a recent report by Shaul et al. (28) of increased gene and protein expression of the endothelial NOS isoform (type III) in whole lung of adult rats after prolonged in vivo exposure to hypoxia. There are several possible explanations for this

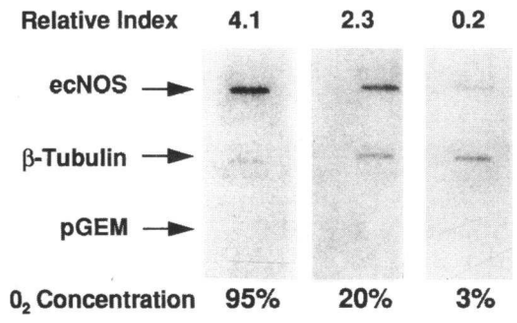

Figure 5. A representative nuclear run-on assay showing the effects of oxygen concentration on transcriptional activity of cNOS and β-tubulin gene at 12 h. Band intensities of cNOS were normalized to β-tubulin for two separate experiments (relative intensity). Specificity was determined by lack of hybridization to the pGEM cDNA.

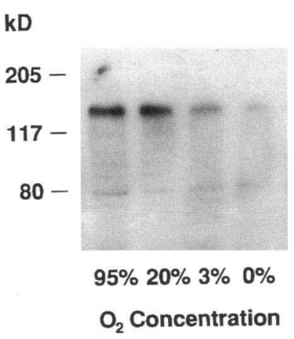

Figure 6. Western blots (50 μg/lane) showing the effects of oxygen concentration on cNOS protein levels after 72 h. This is representative of three separate experiments.

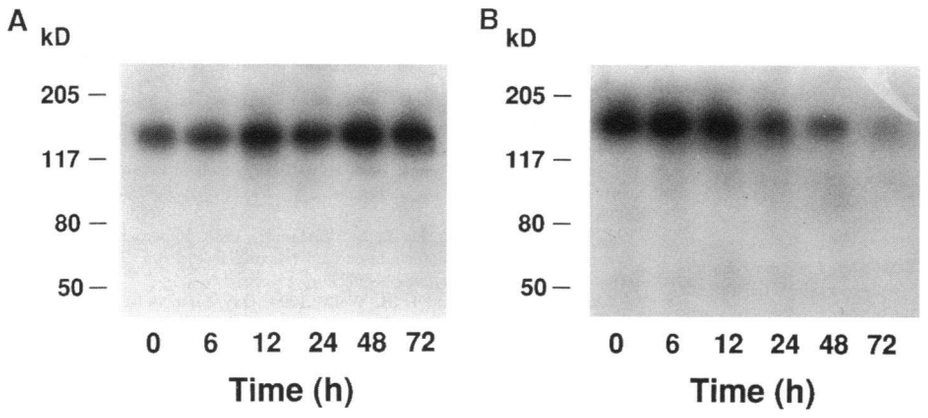

Figure 7. Western blots (50 μg/lane) showing the time-dependent effects of (*A*) hyperoxia (95% O_2) and (*B*) hypoxia (3% O_2) on cNOS protein levels. This is representative of two separate experiments.

discrepancy, aside from the duration of hypoxia. As pointed out by the authors, the exact cellular localization of the increase in NOS III mRNA and protein was not known and could have been from a source other than the endothelium (28). Furthermore, other variables may also be operative in a hypoxic in vivo model. For example, shear stress, which may be increased in the hypoxic pulmonary vasculature, has been shown to upregulate endothelial NOS expression (29). Further studies are needed to sort out the relative importance of the various cellular sources of NO production in the lung and their respective stimuli.

Regulation of gene expression by O_2 tension has been described for other genes, with upregulation at either extreme of O_2 tension according to the gene studied. For example, upregulation by hypoxia has been described for the erythropoietin (30), PDGF-β chain (31), endothelin (32), and the xanthine dehydrogenase (16) genes. The endothelium was the cellular source for all cases except for the erythropoietin gene (human hepatoma cell line). In some of these cases, such as erythropoietin, O_2 affected transcription of the gene as well as mRNA stability (30). Gene upregulation by hyperoxia, on the other hand, has been shown for the basic fibroblast growth factor and one of its receptor genes in the lung, although the specific cellular source was not identified (33).

In addition to its regulation by O_2 tension, cNOS mRNA expression was found to be increased in cycloheximide-treated cells as compared with counterpart untreated cells exposed to the same O_2 concentration (3, 20, and 95% O_2). This suggests inhibition by cycloheximide of short-lived protein(s) with RNase activity, resulting in increased cNOS mRNA stability. This so-called superinduction by cycloheximide has also been proposed for other genes (e.g., the *c-myc* [34], *c-jun* [35], IL-2 [36], and xanthine dehydrogenase [16] genes) and suggests that these genes along with the cNOS gene are negatively regulated under basal conditions.

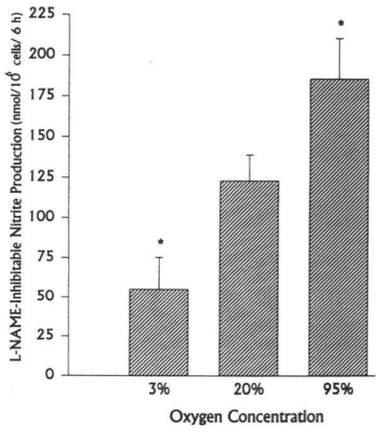

Figure 8. Effect of oxygen concentration on L-NAME–inhibitable nitrite production from EC. This is representative of three separate experiments. Asterisks indicate $P < 0.05$ vs. 20% O_2 ($n = 6$ for each exposure).

Finally, the response of cNOS enzyme activity to changes in O_2 tension was also tested. By measuring the L-NAME–inhibitable portion of the total nitrite produced by normoxic EC as a reflection of NO production, a release of 123 nmol/10^6 cells/6 h was determined, which is comparable with basal NO release reported for another source of cultured EC (37). There was a stepwise threefold increase in NO production from hypoxia (3% O_2) to hyperoxia (95% O_2) which is consistent with the changes in cNOS mRNA and protein levels obtained at a similar exposure time.

It is noteworthy that cNOS gene expression and activity have been found to be significantly increased in growing compared with growth-arrested bovine aortic EC (38). Since our studies were all conducted on confluent EC and there were no significant differences in cell numbers after various O_2 exposures, it is unlikely that differences in cell growth would account for the results obtained.

In conclusion, we postulate that the regulation of cNOS expression by O_2 tension may play an important physiologic role in modulating pulmonary vascular tone and perhaps the integrity of the vessel wall. Aside from being a vasodilator, NO may act as an inhibitor of vascular smooth muscle cells (39). NO has been shown to reduce pulmonary vascular remodeling and protect against the development of pulmonary hypertension in chronically hypoxic rats given continuous inhalation of NO (40). In addition, NO has been shown to downregulate the expression of vasoconstrictors and growth factors from vascular endothelium exposed to normoxia or hypoxia (41). Therefore, the decrease in cNOS expression and activity in hypoxia, as shown in our study, might shift the balance in favor of vasoconstrictors and growth factors, thereby contributing to the development of pulmonary hypertension and vascular remodeling produced by chronic hypoxia.

Acknowledgments

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