Hypoxia Induces Endothelin Gene Expression and Secretion in Cultured Human Endothelium

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Abstract

Hypoxia in vivo is associated with constriction of the distal vasculature in the lung. Uniquely situated at the interface between blood and the vessel wall proper, the vascular endothelium may release vasoactive mediators in the setting of hypoxia. Endothelin-1 is a potent vasoconstrictor released by endothelial cells that could function as a paracrine regulator of vascular tone. We found that physiologic low oxygen tension (Po_2 = 30 Torr) increased endothelin secretion from cultured human endothelial cells four to eightfold above the secretion rate at ambient oxygen tension. This increase in secretion was accompanied by a corresponding increase in the transcriptional rate of the preproendothelin gene resulting in increased steady-state mRNA levels of preproendothelin. In contrast, the transcription of a number of other growth-factor–encoding genes, including transforming growth factor-β, was unaffected by hypoxia. Endothelin transcript production increased within 1 h of hypoxia and persisted for at least 48 h. In addition, the stimulatory effects of low oxygen tension on endothelin mRNA levels were reversible upon reexposure to 21% oxygen environments. These findings suggest a role for endothelin in the control of regional blood flow in the vasculature in response to changes in oxygen tension. (*J. Clin. Invest.* 1991. 88:1054–1057.) Key words: vasoconstrictor • gene regulation • vascular tone • transcriptional regulation • oxygen tension

Introduction

Endothelium-derived relaxing and contracting factors play a key role in modulating vascular smooth muscle tone. Abnormalities in vascular smooth muscle tone and proliferation underlie such diseases as atherosclerosis, pulmonary fibrosis, and persistent pulmonary hypertension of the newborn. Endothelial cells are known to synthesize factors with vasoconstrictive activities. Reports indicate that vasoconstriction of isolated arteries in certain systems is endothelium dependent (1, 2), or endothelium enhanced (3). Similarly, vasorelaxation in response to acetylcholine and bradykinin is endothelial dependent (4, 5). A critical balance in the production of these potent vasoactive substances would, therefore, be essential in the maintenance of normal vascular tone.

Endothelin is a recently discovered 21-amino acid peptide which was first isolated from the supernatant fraction of cultured endothelial cells and found to have potent vasoactive properties (6). Endothelin represents one member of a family of structurally related peptides. Human endothelin-1 (ET-1)[1] is derived from a 212-amino acid precursor, preproendothelin, via a 38-amino acid intermediate. Endothelin is constitutively expressed by endothelial cells and physiological responses to endothelin are reported for blood vessels derived from a large variety of different tissues including the heart, kidney, and brain (7). Factors shown to induce endothelin production include thrombin (8), transforming growth factor β, the Ca^{2+} ionophore A23187, and shear stress (6). The potent effect of endothelin on vascular tone, coupled with the rapid clearance of the circulating peptide, suggests a critical role for locally produced peptide in the regulation of regional blood flow. For the vasculature of the lung, as well as for certain systemic arteries (1), hypoxia is a potent physiologic inducer of local vasoconstriction. We therefore hypothesized that hypoxia might mediate its effects by regulating the release of vasoactive substances, such as endothelin, from endothelial cells. In the studies reported here, early passage cultured endothelium was used to determine the effects of oxygen tension on endothelin production by these cells.

Methods

Cell culture and conditions for achieving hypoxia. Primary cultures of human umbilical vein endothelial cells were subcultured on gelatin-coated plates in the presence of heparin (15 U/ml) and endothelial cell growth supplement (Sigma Chemical Co., St. Louis, MO) in M199 medium (Gibco Laboratories, Grand Island, NY) with 20% FCS (HyClone Laboratories, Logan, UT) at 37°C, 5% CO_2 in a humidified incubator. Cells were passaged by trypsinization with 1% trypsin/EDTA (Gibco) every 3–4 d. Endothelial cells at confluence were exposed to low oxygen tensions in air-tight chambers flushed with preanalyzed gas mixtures at 37°C. The desired oxygen mixture (1% oxygen, 5% CO_2, 94% N_2) was preanalyzed (Northeast Air Gas, Inc., Manchester, NH) and infused periodically into air-tight incubators with inflow and outflow valves (Billups-Rothenberg Inc., Del Mar, CA) at a flow rate of 3 liter/min. The Po_2 was measured in the media at multiple time intervals. We found a steady drop in Po_2 from 150 Torr to 60 Torr by 6

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h. The Po$_2$ reached a nadir of 30 Torr between 12 and 18 hours of 1% oxygen infused in the environment and did not fluctuate throughout the remaining incubation period.

RNA analysis. Total cellular RNA was prepared by the method of Chirgwin et al. (9), from endothelial cells exposed to various oxygen tensions at confluence. 15 µg of RNA per lane was separated by electrophoresis on 1% agarose gels containing formaldehyde and transferred to nitrocellulose membranes by blotting. The filters were hybridized with a ^{32}P-labeled cDNA probe specific for the human preproendothelin gene (10). The actin probe was the 800-bp PstI fragment of the mouse β-actin gene. Hybridization conditions were carried out as previously described (11). For quantitation, transcript levels of preproendothelin were normalized after densitometry to the levels of actin transcripts, which did not change under the various oxygen environments.

Nuclear run-off analysis. Confluent cultures of endothelial cells exposed to 1% or 21% oxygen for 6, 24, and 48 h were lysed and nuclei isolated as described by Kavanaugh et al. (12). 100 µl of nuclear suspension were incubated with 0.5 mM each of CTP, ATP, and GTP, and 250 µCi of α-^{32}P-labeled UTP (3,000 Ci/mmol) (New England Nuclear, Boston, MA). The samples were phenol/chloroform extracted, precipitated, and resuspended at equal cpm/ml in hybridization buffer (10–20 × 10^6 cpm/ml). Hybridization to denatured probes (1 µg) dot-blotted on nitrocellulose filters was performed at 40°C for 2 d in the presence of formamide. cDNA probes for the endothelin-1, platelet-derived growth factor-A (PDGF-A), sodium-potassium ATPase, and β-actin genes were used.

Radiobinding assay. To quantify the amount of endothelin secreted by endothelial cells under the various oxygen tensions, conditioned medium was centrifuged at the end of the incubation periods and applied to preactivated Sep-Pak C$_{18}$ columns. Samples were eluted with ethanol/H$_2$O/acetic acid (90:9.6:0.4), vacuum lyophilized, and resuspended in assay buffer. Determinations of endothelin were performed with a radiobinding assay as previously described by Marsden et al. (13). This competitive radiobinding assay uses rat glomerular microsomes bearing endothelin-specific receptors as the binding fraction and [^{125}I]-ET-1 as the radioactive tracer.

Results

Hypoxia increases preproendothelin mRNA levels in endothelial cells. Primary cultures of human umbilical vein endothelial cells were exposed to a hypoxic atmosphere with Po$_2$ = 30 mmHg (1% oxygen) or to an ambient (21% oxygen) environment. Northern blot analysis of endothelial cell RNA after hybridization with a cDNA probe for preproendothelin-1 showed a marked increase in preproendothelin steady state mRNA levels in cells exposed to low oxygen tensions for 24 or 48 h, as compared to 21% oxygen conditions (Fig. 1 *A*). Endothelin transcript levels increased twofold within 1 h of exposure to hypoxia and approximately six to eightfold by 48 h (Fig. 1 *B*). Return of preproendothelin mRNA to the baseline levels of the normoxic controls was observed within 24 h of reexposure of cells to a 21% oxygen environment (Fig. 1 *C*).

Hypoxia increases the transcriptional rate of endothelin-1. To examine mechanisms by which hypoxia increased endothelin transcript levels, the half-life of the endothelin transcript was determined in the hypoxic and normoxic conditions by actinomycin-D chase experiments. The half-life of the endothelin transcript was ~30 min under both conditions (data not shown). Hence differential stability of preproendothelin mRNA could not account for the increase in steady-state levels found under varying low oxygen environments. Nuclear run-off analyses were carried out to determine the transcriptional rate of the endothelin gene under hypoxic as compared to nor-
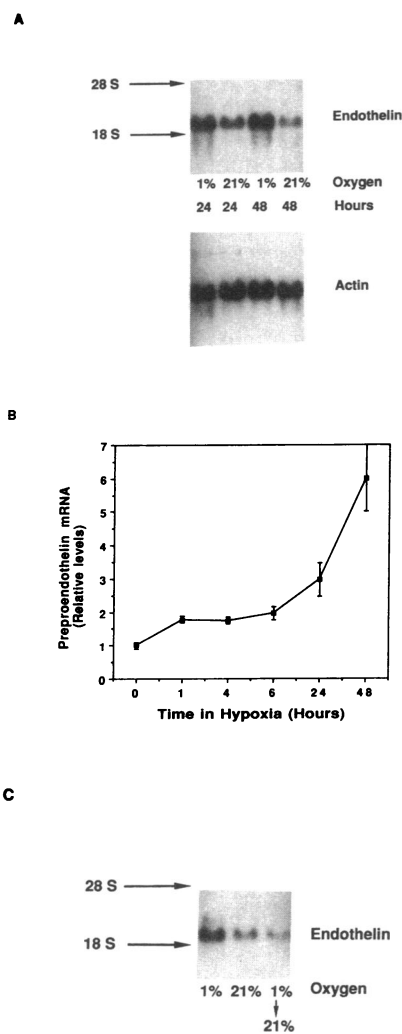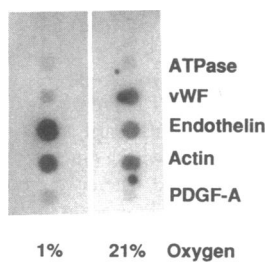
Figure 1. Regulation of preproendothelin mRNA levels by oxygen tension in human umbilical vein endothelial cells. (*A*) Physiologic levels of hypoxia increase preproendothelin mRNA levels in endothelial cells. Early passage human umbilical vein endothelial cells from at least three different donors were grown to confluence in 1% gelatin-coated 10-cm diameter tissue culture plates containing 5 ml of culture media (11). The cells were allowed to reach confluence at 37°C, 5% CO$_2$ and were then placed in a 21%, or 1% oxygen environment with 5% CO$_2$ and the balance N$_2$, for a period of 24 or 48 h. At the end of the 24- and 48-h incubation periods, total cellular RNA was isolated by the method of Chirgwin et al. (9). 15 µg of RNA per lane were separated by electrophoresis on 1% agarose gel containing formaldehyde and transferred to nitrocellulose membrane by blotting. The filter was hybridized with a ^{32}P-labeled cDNA probe specific for the human preproendothelin gene (10). Hybridization conditions and stringent washes were carried out as described previously (22). After autoradiography, the filter was stripped and rehybridized with a β-actin specific probe. (*B*) Time course of preproendothelin mRNA induction by hypoxia. Endothelial cells were fed with growth medium at least 12 h before exposure to hypoxia. Total RNA was extracted at time 0, and after 1, 4, 6, 24, and 48 h of exposure of cells to hypoxia, separated on a formaldehyde gel, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled ET-1 probe. The partial pressure of oxygen in the medium over the cells was 60–80 Torr for the first 6 h and decreased to 30 Torr by 24 h, where it remained for the duration of this experiment. Relative preproendothelin transcript levels are plotted against time of exposure of cells to hypoxia. The preproendothelin mRNA levels at 21% oxygen (time 0) were arbitrarily made equal to 1. The filter was stripped and rehybridized with a β-actin specific probe, followed by an ATPase specific probe. There were no changes in the transcript levels of either β-actin or ATPase genes in response to oxygen tension (not shown). (*C*) The effect of hypoxia on endothelin is reversible. Total cellular RNA was harvested from endothelial cells grown in 1% or 21% oxygen environments for 24 h (lanes *1* and *2,* respectively), and from cells exposed to 1% oxygen for 24 h followed by 21% oxygen for 24 h (lane *3*). Cellular RNA was isolated and transferred to nitrocellulose and the blot was hybridized with a ^{32}P-labeled ET-1 specific probe and then stripped and rehybridized with a β-actin specific probe. β-actin transcript levels remained the same under all conditions (not shown).
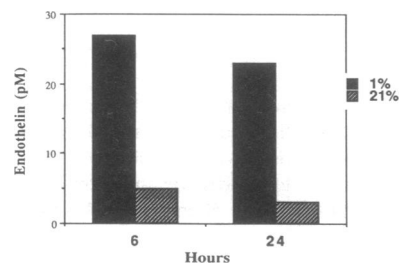
moxic conditions. Transcriptional rates were determined at 6, 24, and 48 h after exposure to 21% or 1% oxygen (Po$_2$ = 30 mmHg) atmosphere. Transcriptional rates of other genes expressed by endothelial cells such as vWF, sodium-potassium ATPase, PDGF-A, and β-actin were analyzed for comparison. In five separate experiments, hypoxia stimulated transcription of the endothelin gene an average of four to eightfold above the rate observed at 21% oxygen (Fig. 2). The transcriptional rates of the ATPase, vWF, and PDGF-A genes were not increased by hypoxia. In two out of five experiments, the gene for β-actin was transcribed at a rate two to three times higher in cells exposed to hypoxic compared to normoxic conditions. (The steady-state mRNA levels of β-actin, however, remained constant under all oxygen environments.) The hypoxia-induced increase in preproendothelin mRNA in human umbilical vein endothelial cells is therefore predominantly due to increased gene transcription as opposed to enhanced stability of its mRNA transcript.

Hypoxia-mediated increases in endothelin secretion. To determine whether this four to eightfold increase in gene transcription resulted in corresponding increases in endothelin protein secreted by endothelial cells, a radioligand binding assay was used to quantitate the endothelin content in conditioned media. After exposing endothelial cells to hypoxia, conditioned media were collected at 1-h, 3-h, 6-h, 12-h, and 24-h intervals in parallel with media from cells exposed to ambient atmospheres. The media were assayed for endothelin activity as described under Methods, and compared between the two conditions at each of the indicated time points. A representative result from four independent experiments is shown on Fig. 3. The amount of secreted endothelin increased four to eightfold when cells were incubated in low oxygen atmospheres as compared to 21% oxygen conditions. This effect was demonstrable within 6 h of exposure to hypoxia. Incubation periods shorter than 6 h yielded no detectable differences in endothelin secretion between the two oxygen environments. It is possible that differences in secretion of endothelin occur substantially faster than 6 h, but the constraints of our system are such that enough endothelin must be secreted by 10^6 cells to change the concentration in 5 ml of overlying medium before it can be detected. Although the radioreceptor assay used here cannot distinguish among the different endothelin isotypes (ET-1, -2, -3), the cDNA probe used in the hybridization experiments recognizes

Figure 3. Hypoxia increases the secretion of endothelin from cultured endothelial cells. Media conditioned by human umbilical vein endothelial cells under normoxic (21% oxygen; *hatched bars*) or hypoxic (1% oxygen; *solid bars*) conditions were collected at the indicated times. Determinations of endothelin were performed with a radiobinding assay as previously described (13). Recovery of ET-1 was ≥ 90%. Inter- and intraassay variability are 11.7% and 6.0%, respectively. After removal of the conditioned media, the cells were lysed and total protein was quantified to normalize for any differences in cell survival. Equal amounts of total cellular protein were obtained under hypoxic and normoxic conditions for each time point, suggesting that this period of hypoxia had no toxic effects on the endothelial cells. Endothelin production is shown as endothelin concentration in 5 ml of medium produced/time.

only the gene for ET-1 and does not cross-react with the others. In addition, the vascular endothelium appears to express ET-1 only (14). Therefore, hypoxia-induced increases in endothelium production by endothelial cells are due to increases in ET-1.

Discussion

Endothelin is a potent vasoconstrictor and also a mitogen for fibroblasts and smooth muscle cells (15, 16). Increases in its secretion from endothelial cells can, therefore, increase the contractile and proliferative state of the underlying fibroblasts and smooth muscle cells. Hypoxia has been reported to have a variety of effects on endothelial cells, ranging from alterations in protein synthesis to changes in gene transcription (17–22). For example, Vender et al. (18) reported the release of a unique smooth muscle cell mitogen from bovine pulmonary endothelial cells exposed to hypoxia. Humphries et al. (19) showed that hypoxia causes a decrease in the secretion of heparan sulfate proteoglycan from cultures of bovine pulmonary artery endothelial cells. We have reported that PDGF-B chain gene expression is dramatically increased in endothelial cells exposed to a narrow and physiologic range of low oxygen tension (Po$_2$ = 20–45 mmHg) (22).

Hypoxia also has been reported to result in the release of vasoactive substances from whole vessels. Rakugi et al. (23) reported a stimulation of endothelin release of 70% above baseline in response to hypoxia in a mesenteric artery perfusate system. Vanhoutte et al. (24) using a bioassay system also found evidence of endothelin-like mediated vasoconstriction in response to hypoxia. In the studies reported here, hypoxia was found to increase endothelin production by endothelial cells principally by increasing the transcriptional rate of the gene within 1 h of exposure to low oxygen tension. The maximal increase was four to eightfold above the baseline rate of transcription present at ambient atmospheres. A comparable increase was shown for the amount of total endothelin protein secreted by these cells within 6 h after hypoxic exposure. The endothelial cells continued to proliferate during exposure to

Figure 2. Hypoxia increases endothelin gene transcription. Endothelial cells at confluence were exposed to 1% or 21% oxygen environments for 6, 24, and 48 h as described in the legend to Fig. 1. Nuclei were isolated as described previously (15, 21), and in vitro transcription was allowed to resume in the presence of α-^{32}P-UTP. Equal amounts of ^{32}P-labeled, in vitro transcribed RNA were hybridized to denatured cDNA for the endothelin-1, PDGF-A, sodium-potassium ATPase, and β-actin genes, which had been previously immobilized on nitrocellulose filters. Shown are representative autoradiograms from nuclear transcription after 6 h of exposure to 1% or 21% oxygen, from five separate experiments. Similar results are obtained after 24 or 48 h of exposure to hypoxia.

hypoxia, and continued to express transcripts for other growth factors like transforming growth factor β, PDGF-A, basic fibroblast growth factor, and structural proteins like β-actin (22). Furthermore, endothelin production returned to baseline levels upon reexposure of cells to normoxia suggesting that the cellular response to hypoxia was not mediated by cellular toxicity. The increase in gene transcription among the growth factors tested was limited to the vasoconstrictors (endothelin and PDGF-B) produced by endothelial cells.

In contrast to PDGF-B which was induced 24 h after hypoxic exposure, however, increases in endothelin production occurred within 1 h of hypoxia and lasted for a period of at least 2–3 d (longer exposure times were not examined). Indeed, not only is the response time of the endothelin gene to hypoxia much faster than for the PDGF-B gene, but its expression is increased at milder degrees of hypoxia. The physical constraints of the experimental systems we employ in these studies result in a substantial delay period before the oxygen tension in the media bathing the endothelial cells equilibrates with the surrounding gas mixture. As a result, the Po_2 in the media is about 60–80 Torr after 1 h of exposure of cells to 1% oxygen atmospheres. Yet, this period of time and mild degree of hypoxia is sufficient to increase endothelin transcript levels. A more severe degree of hypoxia, namely a Po_2 of 20–45 Torr, was necessary to induce the PDGF-B gene.

The endothelial cells exhibit a two-phase response to hypoxia stimuli in their secretion of vasoactive agents. The sensitive and rapid regulation of endothelin expression in response to environmental oxygen makes it a likely candidate as an important regulator of regional blood flow in response to acute changes in oxygen tension. Hypoxia-induced contractions of pulmonary artery strips occur at least 2–6 h after an adaptation at Po_2 of 20–40 Torr (1). Factors such as endothelin that are secreted within 6 h of hypoxia could mediate such vasoconstrictive events. PDGF-B, with its later induction requiring more severe degrees of hypoxia, may then be a more important modulator of the smooth muscle hypertrophy and architectural remodeling occurring in chronic hypoxia. Both processes, vasoconstriction and smooth muscle cell hyperplasia, are critical to the pathogenesis of diseases such as atherosclerosis, pulmonary fibrosis, and pulmonary hypertension.

Acknowledgments

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