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Lipopolysaccharide and interleukin 1 augment the effects of hypoxia and inflammation in human pulmonary arterial tissue

(pulmonary hypertension/nitric oxide synthases/inflammation/hypoxia)

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ABSTRACT **The combined effects of hypoxia and interleukin 1, lipopolysaccharide, or tumor necrosis factor α on the expression of genes encoding endothelial constitutive and inducible nitric oxide synthases, endothelin 1, interleukin 6, and interleukin 8 were investigated in human primary pulmonary endothelial cells and whole pulmonary artery organoid cultures. Hypoxia decreased the expression of constitutive endothelial nitric oxide synthase (NOS-3) mRNA and NOS-3 protein as compared with normoxic conditions. The inhibition of expression of NOS-3 corresponded with a reduced production of NO. A combination of hypoxia with bacterial lipopolysaccharide, interleukin 1β, or tumor necrosis factor α augmented both effects. In contrast, the combination of hypoxia and the inflammatory mediators superinduced the expression of endothelin 1, interleukin 6, and interleukin 8. Here, we have shown that inflammatory mediators aggravate the effect of hypoxia on the down-regulation of NOS-3 and increase the expression of proinflammatory cytokines in human pulmonary endothelial cells and whole pulmonary artery organoid cultures.**

The pathogenesis of pulmonary hypertension is unknown. However, both experimental and clinical evidence indicate that hypoxia or inflammation dramatically aggravates the extent of pulmonary hypertension, e.g., during acute bacterial infection in patients with chronic obstructive pulmonary disease. Experimental evidence suggests endothelial nitric oxide production as the main regulator of vascular tone in pulmonary arteries. Nitric oxide (NO) is mainly produced by three isoforms of NO synthases (NOS 1–3), two of which are constitutively present in neuronal or endothelial cells (NOS-1 or NOS-3, respectively) (1). In human umbilical vein endothelial cells, hypoxia was found to be responsible for both a decrease of human NOS-3 and an increase of human endothelin 1 (ET-1) mRNA (2). Conversely, exposure of rat lungs to hypoxia for more than 1 week caused an increase of both NOS-3 mRNA and protein leading to a doubled production of NO (3). In short-term cultures using human aortic endothelial cells, inflammatory mediators have been demonstrated both to increase the mRNA of the inducible isoform of nitric oxide synthases (iNOS or NOS-2) and to decrease the amount of NOS-3 mRNA, which may imply a major influence of NOS-2 on the regulation of vascular tone during inflammatory states (4). NO has been shown to down-regulate the expression of vasoconstrictors and growth factors such as ET-1, interleukin 1 (IL-1), and platelet-derived growth factor in human umbilical vein endothelial cells (5). Similarly, proinflammatory cytokines were shown to induce the synthesis of NO, suggesting a crucial role of inflammatory mediators in the regulation of arterial tissues (6). Analogously, hypoxia stimulated the induction of major vascular growth factors such as ET-1 (5) and platelet-derived growth factor B (PDGF-B) (8). To evaluate the influence of inflammation and/or hypoxia on the regulation of NO biosynthesis, we characterized the effect of both conditions on the regulation of NO synthases and growth factors as well as on the production of NO in human pulmonary artery endothelial cells (HPAEC) and pulmonary artery organoid cultures obtained from peripheral pulmonary arteries.

METHODS

Reagents and Antibodies. Recombinant human IL-1β was purchased from Endogen (Cambridge, MA). Recombinant human tumor necrosis factor α (TNF-α) was from R & D Systems, and lipopolysaccharide (LPS) (from *Pseudomonas aeruginosa*) was obtained from Sigma. Monoclonal antibody (mAb) anti-NOS-3 was purchased from Transduction Laboratories (Lexington, KY) and anti-IL-6 mAb was from Bender MedSystems (Vienna).

Organoid Cultures of Pulmonary Arteries. Preparation of pulmonary arteries was performed following the protocol of Holt *et al.* (9). In brief, after removal of the lung during pneumonectomy, peripheral pulmonary arteries were dissected, one slice of each artery was fixed with 4% freshly depolymerized paraformaldehyde (Merck) for assessment of basic conditions, and the remaining arteries were placed in Medium 199 at 4°C (1 mg/ml streptomycin, 600 μg/ml penicillin, 50 units/ml heparin; Sigma) and processed within 15 min. Arteries were opened along their upper aspect, and atraumatically cut into pieces of 5 × 5 mm. The pieces were placed with the endothelial surface on top and were challenged under normoxic (21% O_2/5% CO_2) or hypoxic conditions (3% O_2/5% CO_2) in the presence or absence of the proinflammatory stimulators for a period of time, lasting up to 72 h. After challenge, the arterial pieces were fixed in 4% paraformaldehyde for 20–60 min, washed in phosphate-buffered saline (PBS; pH 7.4), dehydrated by a series of increasing ethanol concentrations, and embedded in Paraplast (Sherwood Medical Industries, Athy, Ireland).

Cell Isolation and Culture. HPAEC was isolated from small dissected segments of the arteria pulmonalis that was removed with the lung during pneumonectomy. Following incubation

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Abbreviations: HPAEC, human pulmonary artery endothelial cells; ET-1, endothelin 1; IL, interleukin; TNF-α, tumor necrosis factor α; NOS-2, inducible nitric oxide synthase isoform; NOS-3, endothelial constitutive nitric oxide synthase isoform; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide.

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with collagenase (Sigma), the endothelial cells were separated using the magnetic beads method (10). Single-donor HPAECs were obtained from Clonetics (San Diego). Indirect fluorescent antibody staining for endothelial factor VIII was uniformly positive for both cell lines. Cells were cultured and challenged in endothelial basal medium (Promo Cell, Heidelberg) supplemented with 20% fetal calf serum (GIBCO/BRL), heparin (Sigma), and endothelial cell growth supplement (Becton Dickinson). For protein and mRNA isolation, cells were grown to confluence on gelatin-coated 75 cm^2 tissue culture flasks (Falcon, Becton Dickinson) and used between passages 5–8. All experiments were performed as triplicate.

***In Situ* Hybridization.** All chemicals for prehybridization and hybridization were purchased from Sigma unless stated otherwise. Sections (5 μm thick) were dewaxed, and endogeneous alkaline phosphatase was blocked by 0.5% acetic anhydride (0.1 M Tris, pH 8, for 10 min). Digestion of proteins was performed in 0.1% pepsin (20 min at 37°C) under microscopic control to reduce loss of tissue. Excess aldehyde groups were blocked in 0.2% glycine in PBS (10 min), followed by preincubation in 10 μl hybridization mix (15 min at 60°C) in a humidity chamber with an atmosphere made up by 50% formamide in 2× standard saline citrate (SSC). After preincubation, 10 μl hybridization mix containing 50 ng of a digoxigenin-labeled riboprobe for IL-6 (hIL6/pGEM4, *Nhe*I digest, T7 polymerase-amplified) or NOS-3 (pHuNOSendoPM21, *Xba*I digest, T7 polymerase-amplified) was added (90°C for 10 min) and incubated at 60°C overnight. Following digestion of nonhybridized RNA with RNase A (100 μg/ml for 30 min at 37°C), anti-digoxigenin Fab fragments coupled to alkaline phosphatase (1:500; Boehringer Mannheim) were added for 12 h (4°C). Finally, 100 μl substrate [0.45% nitroblue tetrazoloum (NBT) and 0.35% X phosphate/5-bromo-4-chloro-3-indolyl phosphate (BCIP) in alkaline buffer, Boehringer Mannheim] was added. Development lasted between 4 and 24 h. Photographs were taken on a Nikon FXA 118 light microscope. Hybridization control was performed using doubled concentration of corresponding sense probes. For check of endogenous alkaline phosphatase activity, alkaline phosphatase-coupled antibody was omitted in the detection protocol. Control of specificity was done by incubation without digoxigenin-labeled RNA. In all cases, nonspecific signals were not observed.

Immunological Detection of IL-6, NOS-2, and NOS-3. Sections on silane coated glass slides were dewaxed and rehydrated as described by Bromley *et al.* (11). The slides were then preincubated in PBS supplemented with 10% fetal calf serum and 0.05% Tween 20, kept overnight at 4°C with anti-IL-6 (Bender MedSystems), anti-NOS-2, and anti-NOS-3 mAb (Transduction Laboratories; final dilutions 1:10 and 1:500, respectively), washed again (40 min), and incubated with rabbit anti-mouse Ig-Fab fragments conjugated to alkaline phosphatase (Boehringer Mannheim). Signal development was performed as described before (12) using NBT/BCIP as alkaline phosphatase substrate (Boehringer Mannheim).

Detection of Cytokine Transcription by Reverse Transcriptase–PCR. cDNA synthesis was performed using the 1st-Strand cDNA Synthesis Kit [CLONTECH; 1 μg total RNA, 20 pmol oligo$(dT)_{18}$ primer, 4 μl 5× reaction buffer (50 mM Tris·HCl, pH 8.3/75 mM KCl/3 mM $MgCl_2$), 0.5 mM of each dNTP, 1 unit/μl RNase inhibitor, and 200 units/μg RNA Moloney murine leukemia virus reverse transcriptase] in a total volume of 20 μl. Two microliters of a 1:5 dilution were used for PCR. PCR was performed in a total volume of 50 μl using Master Mix (Perkin–Elmer) containing 10 μl dNTP mix (0.2 mM final; Boehringer Mannheim), 0.4 μM of specific primers [for IL-6: 5′ primer (ATG AAC TCC TTC TCC ACA AGC GC) and 3′ primer (G AAG AGC CCT CAG GCT GGA CTG)], and 1 μl premixed human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplimers (CLONTECH) as constitutive control. Twenty-eight cycles of a hot start PCR were performed on a Perkin–Elmer Thermal Cycler model 480

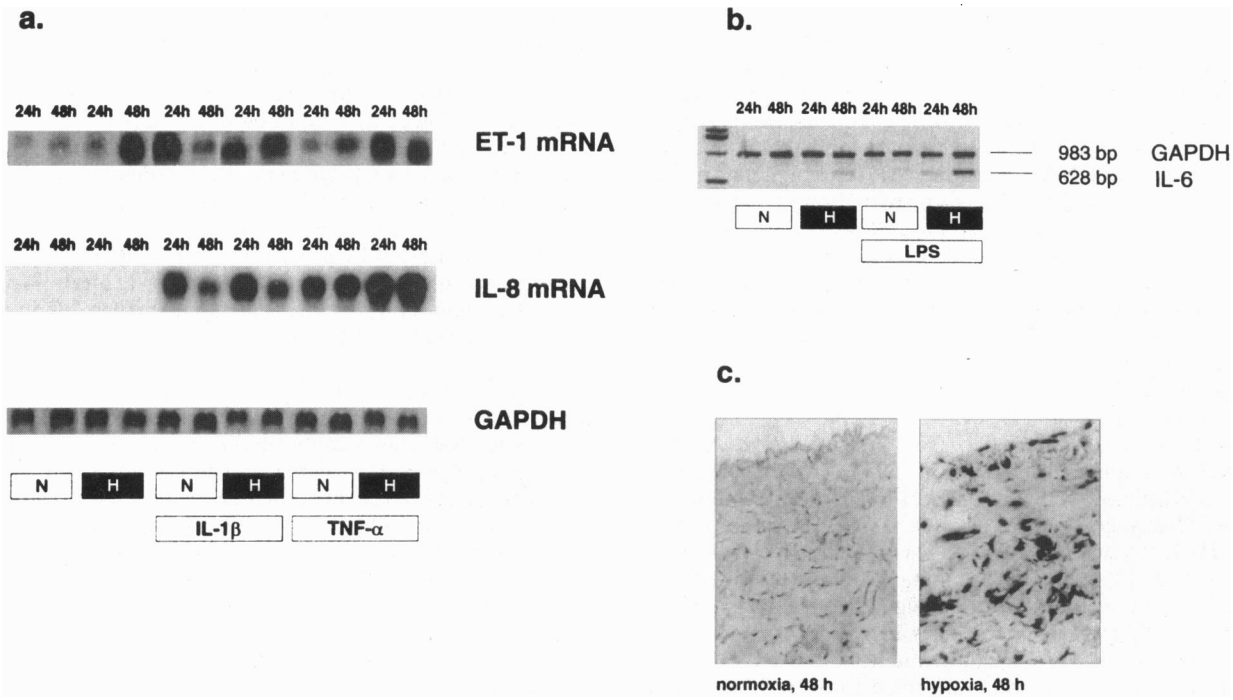

FIG. 1. (*a*) Transcription of ET-1 and IL-8 mRNA by HPAEC was determined by Northern blot analysis in monolayer cultures (N, normoxia; H, hypoxia). Transcription of GAPDH gene was used as constitutive control. Cells were challenged for 24 and 48 h under either normoxic or hypoxic conditions in the presence and absence of IL-1β (2.5 ng/ml) or TNF-α (2.5 ng/ml). (*b*) Assessment of IL-6 and GAPDH in whole organoid cultures by combined reverse transcriptase-PCR. IL-6 and GAPDH were coamplified and electrophoretically separated (GAPDH = 983 bp; IL-6 = 628 bp). (*c*) Detection of IL-6 mRNA by *in situ* hybridization following 48 h of normoxia or hypoxia in human pulmonary artery whole organ cultures. (×300.)

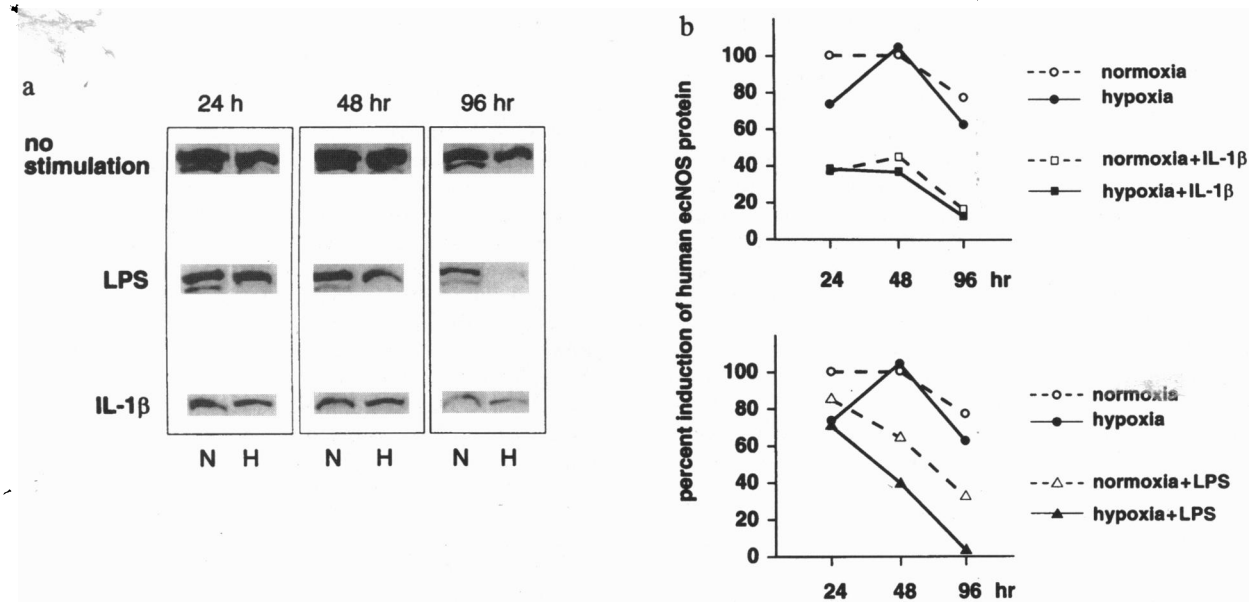

FIG. 2. (*a*) Assessment of NOS-3 proteins after 24, 48, and 96 h in HPAEC monolayer cultures by Western blot. Pulmonary endothelial cells were grown on gelatin-coated flasks and challenged under normoxic (N) or hypoxic (H) conditions with and without LPS (0.1 μg/ml). Two bands of 155 kDa and 140 kDa were detectable. Densitometry was performed using the IMAGEQUANT system (Molecular Dynamics). (*b*) The time course of a representative experiment is given in percent of maximum protein expression observed after 24 h normoxia.

(initially 94°C for 2 min), denaturation (94°C for 45 s), annealing (60°C for 45 s), and extension (72°C for 2 min). Afterwards, an additional extension (72°C for 7 min) was allowed. Aliquots (30 μl) were electrophoretically separated on a 1.7% agarose gel (GIBCO/BRL) and visualized by ethidium bromide staining.

Northern Blot Analysis. Total RNA was extracted from HPAEC monolayers using the acid guanidinium thiocyanate-phenol-chloroform extraction as described (13). Aliquots (10–20 μg) of total RNA were size-fractionated by electrophoresis through 1% agarose/7% formaldehyde gels, transferred to nylon membranes (Hybond-N^+, Amersham), and hybridized with a ^{32}P-labeled 2.18-kb *Eco*RI-digested cDNA insert from pHuNOSendo PM21, which was kindly provided by P. A. Marsden (Department of Molecular and Medical Genetics, University of Toronto, Toronto) for NOS-3. For detection of NOS-2 cDNA, we used a 0.817-kb fragment excised by *Hin*cII/*Eco*RI that was kindly provided by Qiao-wen Xie (Cornell University Medical College, New York). The ET-1 cDNA probe of 0.85 kb was excised with *Eco*RI of the pHET91 plasmid, which came from M. Yanagisawa (Howard Hughes Medical Institute, University of Texas, Dallas). An IL-8 289-bp cDNA fragment was made from human endothelial cell RNA by reverse transcriptase-PCR technique as described above with specific primers (5′ primer, 5′-ATGACTTCCAAGCTGGCCGTGGCT-3′; 3′ primer, 5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′) purchased from CLONTECH. A 1-kb IL-6 cDNA fragment subcloned in pGEM4 was a kind gift from T. Kishimoto (Institute for Molecular and Cellular Biology, Osaka University, Osaka). As an internal control, GAPDH cDNA was used. The DNA fragments were labeled with [α-^{32}P]dCTP by random priming. Blots were washed and exposed to a phosphor screen and signals were quantified by using a PhosphorImager (Molecular Dynamics).

Western Blot Analysis. Equal amounts of protein (100 μg) from whole cell extracts were subjected to SDS/PAGE, and transferred to nitrocellulose (Hybond-Enhanced Chemiluminescence, Amersham) by electroblotting (Trans-Blot SD, Bio-Rad). Transfer efficiency was monitored by Ponceau S staining of membranes; after removing the staining solution, the membranes were probed by using 1:2500 dilutions of anti-NOS-3 (mAb). The blots were developed by using the Enhanced Chemiluminescence system (Amersham) according to the manufacturer's instructions.

Measurement of NO Production. The measurement of NO was performed by gas chromatography using electron capture detection based on the method of Tesch *et al.* (14) where the nitrate (NO_3^-) concentration of the sample is quantified by measuring the production of nitrobenzene under strong acidic conditions. Experimental samples were prepared for analysis by firstly removing exogenous Cl^- ions by precipitating with an equal volume of saturated silver sulfate solution. Following centrifugation at 3000 rpm for 5 min, duplicate 100 μl aliquots were mixed with either 50 μl of water or 0.5 M hydrogen peroxide. A 100 μl aliquot of internal standard (1×10^5 dilution of stock nitrotoluene in benzene) was then added to each tube followed by 1 ml of benzene and 500 μl of concentrated sulfuric acid, and the mixture was again rotamixed (3000 rpm for 5 min at 4°C). One microliter volumes were injected automatically into the HP5890 gas chromatograph fitted with a DB-5 fused silica capillary column 30 × 0.32 mm i.d. coated to a film thickness of 1.0 μm. Initial oven temperature was 60°C (2 min), and it was then increased to 100°C at a rate of 5°C per min. This temperature was held for a further 2 min before being increased to 160°C at a rate of 10°C per min. Under these conditions, nitrobenzene and the internal standard nitrotoluene had retention times of 9.1 and 10.6 min, respectively. Quantification of the nitrobenzene generated by the NO_3^- ions was calculated from a calibration curve produced by plotting the peak height ratios of the known concentration of nitrobenzene peak to the nitrotoluene peak against the known concentration of nitrobenzene added to the sample. Nitrite NO_2^- concentrations were calculated by subtracting the concentration found in the experimental samples without hydrogen peroxide from the concentration of the nitrobenzene in the duplicate sample treated with hydrogen peroxide. The calibration curve was prepared within the range of 0–400 μM of added NO_3^- ions.

RESULTS

Hypoxia Stimulates the Expression of Proinflammatory Mediators in HPAECs. Under normoxic conditions, transcription of ET-1 in HPAEC was very low, and IL-8 mRNA could

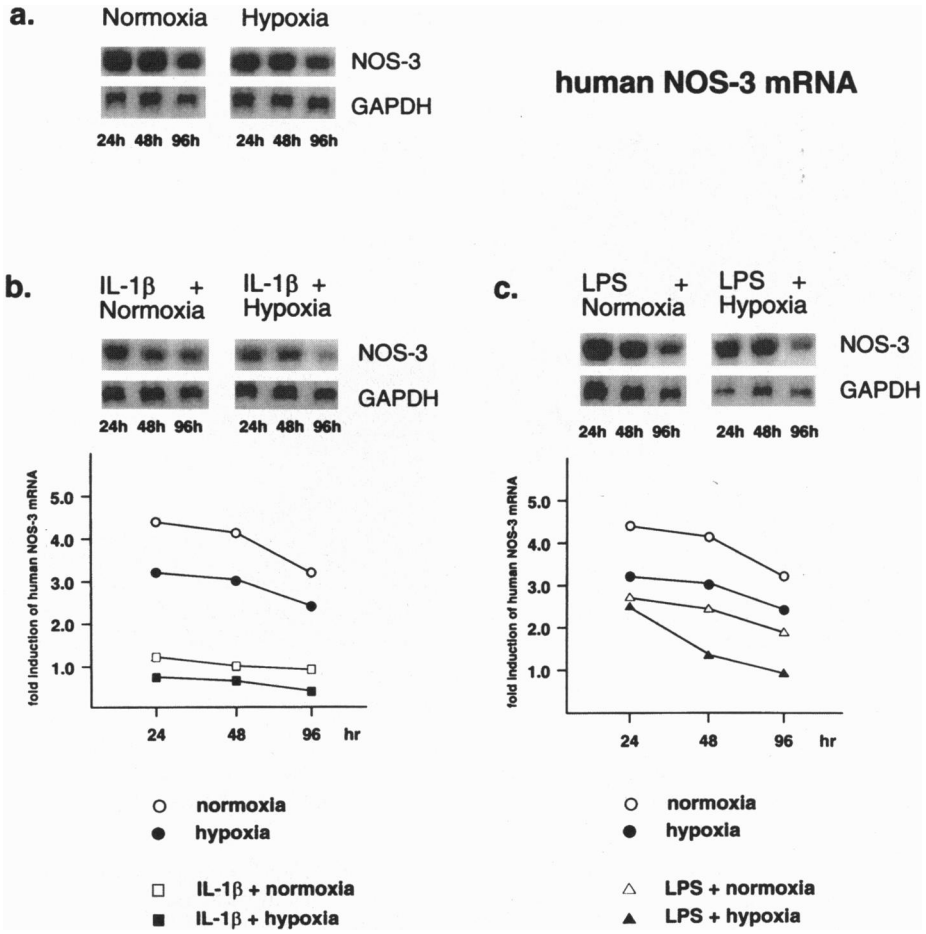

FIG. 3. Representative Northern blot analysis of human NOS-3 in HPAEC. Expression of NOS-3 mRNA was maximal under normoxic conditions without additional stimulation between 0 and 48 h. Transcription of NOS-3 is demonstrated under normoxic and hypoxic culture conditions (*a*), after simultaneous challenge with IL-1β (2.5 ng/ml) and normoxia/hypoxia (*b*), or a combined stimulation with LPS (0.1 μg/ml) (*c*). The transcription of the NOS-3 gene is given between 24 and 96 h. Endothelial cells were used in passages 2–4. GAPDH was used as constitutive control. Quantitative assessment is provided as quotient of induction over constitutive level.

not be detected (Fig. 1*a*). Contrary to IL-8, transcription of ET-1 mRNA significantly increased after stimulation by hypoxia at 48 h. Stimulation of HPAEC with IL-1β caused an 8-fold transcription of ET-1 mRNA, which decreased to nearly normal values after 48 h. However, a combined stimulation by IL-1β and hypoxia significantly prolonged the transcription of ET-1 mRNA up to 48 h. The effect of hypoxia on the transcription of ET-1 or IL-8 was even more pronounced when using TNF-α as the stimulating agent (Fig. 1*a*). TNF-α alone caused a nearly 5-fold increase of IL-8 mRNA as compared with the constitutive level. However, in combination with hypoxia, a more than 10-fold increase over constitutive values could be observed.

Fig. 1*b* demonstrates a typical result of a reverse transcriptase-PCR analysis from whole pulmonary arterial tissues following stimulation with hypoxia or LPS or a combination of both conditions. The upper lanes represent the transcription of the constitutive control gene (GAPDH; 983 bp); the lower lanes demonstrate the influence of normoxia and hypoxia on the transcription of IL-6 mRNA in the presence or absence of additional LPS. Both 48 and 96 h of normoxia did not affect the transcription of the IL-6 gene. Hypoxia increased in a time-dependent manner the amount of IL-6 mRNA. Similarly, LPS alone slightly increased IL-6 mRNA levels after 48 h. The combination of both stimulators led to a significant increase of IL-6 transcription.

These results were confirmed by *in situ* hybridization of whole pulmonary artery organoid cultures (Fig. 1*c*), demonstrating a significant increase of the transcription of IL-6 mRNA following 48 h of hypoxia within the arterial tissue, whereas under normoxic conditions, IL-6 mRNA was undetectable.

Proinflammatory Mediators and Hypoxia Reduce the Expression of NOS-3 Proteins. The expression of the human NOS-3 protein was determined by Western blot at 24, 48, or 96 h of incubation under normoxic or hypoxic conditions in the absence and presence of LPS or IL-1β (Fig. 2). A biphasic effect in the expression of the two NOS-3 proteins of 155 kDa and 140 kDa was noted: while at 24 h, the expression NOS-3 proteins was reduced, it increased after 48 h, and again decreased after 96 h of hypoxia. When tested under normoxic conditions, the addition of LPS or IL-1β strongly decreased the expression of both NOS-3 proteins over time. The inhibitory effect on protein expression by the inflammatory cytokines was further enhanced by hypoxia.

IL-1 and LPS Augment Hypoxia-Induced Down-Regulation of Human Endothelial NO Synthase mRNA in HPAECs. Under normoxia, maximal transcription of NOS-3 mRNA was observed reaching its maximum after 48 h, and decreasing thereafter (Fig. 3a). Addition of IL-1β under normoxic conditions significantly reduced the transcription of NOS-3 mRNA by more than factor of 4 as compared with the normoxic control (Fig. 1*b*). Similarly, LPS caused a time-dependent decrease of NOS-3 transcription, although the effect of LPS was less pronounced than that of IL-1β at all time points (Fig. 1*c*) Hypoxia aggravated both the effects of IL-1 and LPS, leading to a complete down-regulation of NOS-3

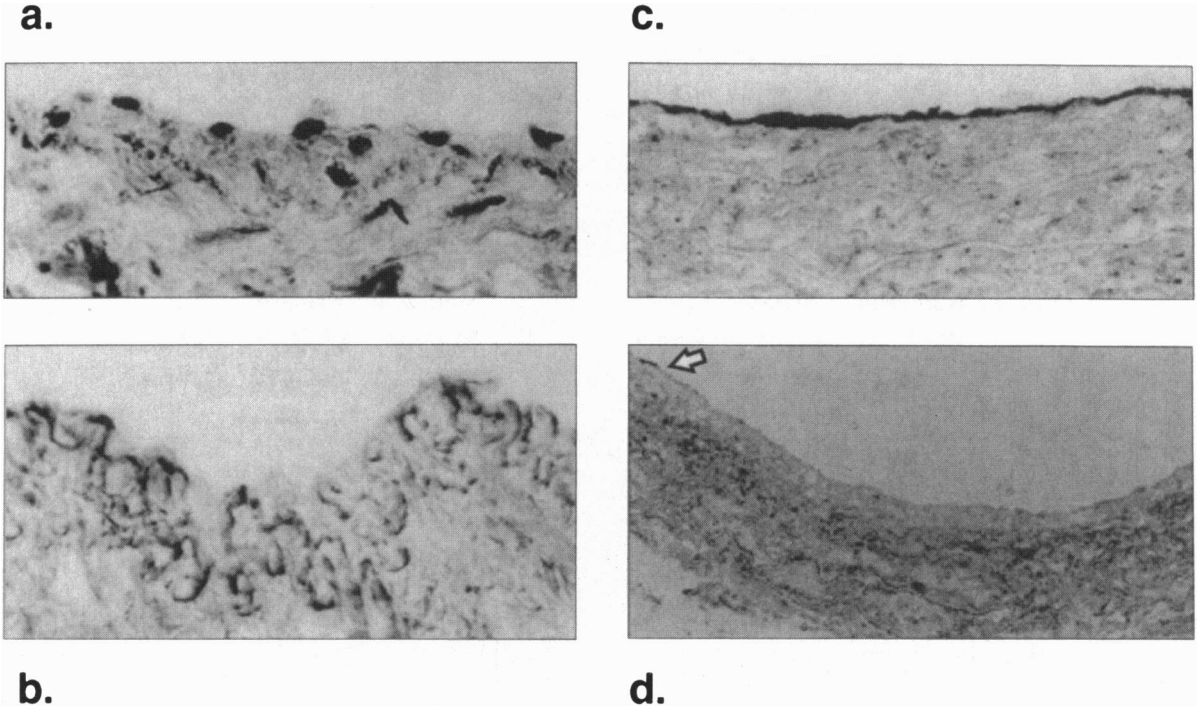

FIG. 4. Expression of NOS-3 in whole pulmonary organoid cultures. Assessment of NOS-3 transcription after 48 h normoxia (*a*) or hypoxia (*b*) by *in situ* hybridization following 48 h of culture. Immunohistochemical detection of NOS-3 following 72 h of normoxia (*c*) and hypoxia (*d*). The arrow indicates a still positive cell after 72 h of culture under low oxygen condition (3% O_2). Oxygen tension within the culture supernatants was 45 mmHg. (*a* and *b*, ×640; *c* and *d*, ×100.)

mRNA after 96 h. Following stimulation with IL-1β, LPS, or TNF-α in the presence or absence of normoxic culture conditions, HPAECs did not express NOS-2 (data not shown).

Assessment of NOS-3 Synthesis in Whole Pulmonary Artery Organoid Culture. Fig. 4 demonstrates the transcription of the NOS-3 gene using *in situ* hybridization technique following 48 h of normoxia (Fig. 4*a*) or hypoxia (Fig. 4*b*) The transcription of NOS-3 mRNA under hypoxic conditions was strongly decreased as compared with normoxia. Analogously, hypoxia (Fig. 4*d*) inhibited the expression of the NOS-3 protein as measured after 72 h in comparison to normoxia (Fig. 4*c*).

Hypoxia and LPS Reduce the Biosynthesis of NO in Long-Term Pulmonary Artery Organoid Cultures. Under normoxic conditions, synthesis of NO was between 170 and 180 μM (100% of basic level). Hypoxia alone decreased the amount of NO produced by the whole pulmonary artery organoid cultures by about 70% after 6 h of incubation, followed by an increase after 12 h and a steady decrease thereafter (Fig. 5). LPS alone caused a dramatic increase of NO synthesis, reaching a maximum concentration of (540%; 325 μM) NO after 6 h; after 6 h, it declined over 48 h to levels 50% below the normoxic control. The combination of hypoxia and LPS caused a significant decrease of NO production already after 12 h reaching basic levels after 36 h.

DISCUSSION

In human pulmonary arterial tissue, a combination of hypoxia with inflammatory mediators caused a complete down-regulation of the expression of NOS-3 that correlated with reduced biosynthesis of NO. Reversely, the combination of hypoxia with proinflammatory agents such as LPS, IL-1β, or TNF-α maximally increased the transcription of ET-1, IL-6, and IL-8.

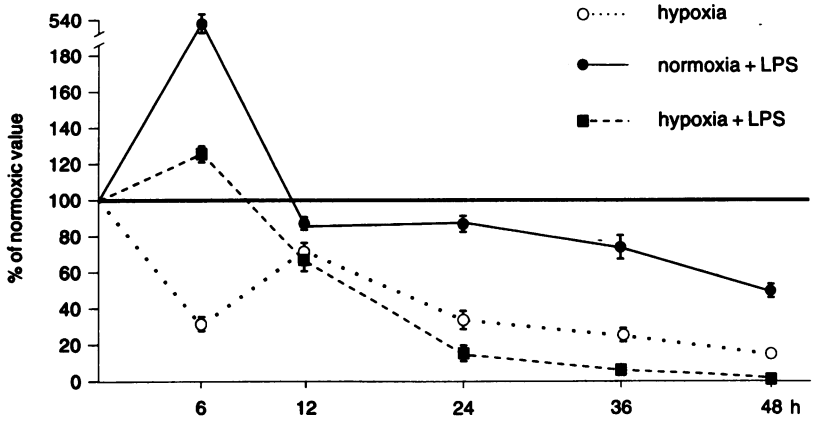

FIG. 5. Determination of NO in the culture supernatants of whole pulmonary artery organoid cultures. Samples were taken after 6, 12, 24, 36, and 48 h of incubation under normoxic or hypoxic conditions with and without LPS (0.1 μg/ml) and were immediately frozen. Samples were assayed by determination of the nitration of benzene to nitrobenzene using gas chromatography. Values are given as percent of the normoxic value (mean ± SD, $n = 3$).

Although the pathogenesis of pulmonary hypertension is unknown, it is well-established that both hypoxia and acute and chronic inflammation are major causes of pulmonary hypertension. This evidenced from the following facts: (*i*) Morphologically, chronic pulmonary hypertension is characterized by the development of media hypertrophy and intima fibrosis indicating inflammatory mechanisms as underlying events of pulmonary hypertension. (*ii*) Evaluation of plexiform lesions in primary pulmonary hypertension revealed intense perivascular inflammatory activity accompanied by the expression of extracellular matrix proteins that were associated with increased growth and differentiation of abnormal endothelial cells (15). (*iii*) Pulmonary hypertension due to hypoxia led to an increased expression of nonphysiological extracellular matrix proteins (16). (*iv*) In rats, pulmonary artery pressure rose significantly due to bacterial infection of the lung caused by pseudomonas aeruginosa (17). The levels of ET-1, which is probably the most potent vasoconstrictor in pulmonary vasculature as well as a mitogen, rose significantly in patients with pulmonary hypertension (18). Moreover, the human adult respiratory distress syndrome is characterized by an intense neutrophilic inflammation of pulmonary tissues accompanied by severe pulmonary hypertension and hypoxia (19), and mediators of inflammation have been demonstrated to aggravate this condition (20).

How could hypoxia and its combination with inflammatory mediators lead to vascular remodeling? Hypoxia was reported to stimulate the transcription of endothelial-derived growth factors such as ET-1, platelet-derived growth factor, and vascular endothelial growth factor in human umbilical vein endothelial cells and whole perfused rat lungs (5, 21). Using human pulmonary artery endothelial cells, we show that hypoxia increases the induction of ET-1, which may at least in part explain the increased growth of vascular smooth muscle cells. This effect was augmented in the presence of IL-1β and TNF-α, both of which have been reported to be induced during acute and chronic inflammation. Furthermore, following stimulation of pulmonary endothelial cells by TNF-α or LPS, hypoxia potentiates the induction of another growth factor, IL-6 (22), and that of IL-8 (23), a chemoattractant frequently found in pulmonary inflammation. It is therefore possible that hypoxia induces increased growth by the synthesis of growth factors such as ET-1. A combination of hypoxia with proinflammatory factors aggravates the cellular changes. Parallel to these events, increased expression of other growth factors, such as IL-6, or inflammatory mediators, such as IL-8, released by paracrine mechanisms, intensify the histopathophysiological feature of pulmonary hypertension.

Endothelial-derived NO is considered to be the main vasorelaxant factor in pulmonary circulation. It has been reported that NO regulates the expression of vasoconstrictors and growth factors by the vascular endothelium under both normoxia and hypoxia (5). We show that within 4 days, the combination of proinflammatory factors and hypoxia are capable of completely nullifying both the expression of endothelial NO synthase and the biosynthesis of NO in human pulmonary endothelial cells and whole pulmonary organoid cultures. At the same time, the transcription of growth hormones such as IL-6, IL-8, or ET-1 frequently produced during inflammation of the lung was significantly increased. Since proinflammatory mediators such as TNF-α are capable of down-regulating NOS-3 mRNA (24), prolonged expression of these mediators could lead to a disability of the pulmonary endothelium to maintain sufficient levels of NOS-3, especially under conditions of increased inflammatory activity. Our observation confirms the inhibition of transcription of NOS-3 during hypoxia observed in human umbilical vein endothelial cells and bovine pulmonary endothelial cells. To unravel the consequences of inflammation with and without hypoxia for NO biosynthesis, we found that hypoxia and inflammation clearly down-regulate the ability of the cells to release NO, which may in part be due to a reduction of substrate availability (25, 26). Despite the early increase of NO production after stimulation with LPS, which could be due to an activation of NOS-2 in the vascular smooth muscle cells or immunocompetent cells of the vascular adventitia since HPAEC did not express NOS-2, both stimuli completely down-regulated the synthesis of NO from pulmonary arteries over a period of 2 days. This is in contrast to a prolonged expression of NOS-3 in cultures of rat lungs exposed to long-term hypoxia (3). However, pathological evaluation of human tissue from individuals with different types of pulmonary hypertension has demonstrated that in all forms, the expression of the constitutive endothelial nitric oxide synthase was significantly lower than in normals (7).

Our data suggest that hypoxia known to be associated with inflammation of the lung leads to the increased production of growth hormones of pulmonary arterial tissue. This effect is potentiated by the action of inflammatory mediators such as LPS, IL-8, and TNF-α frequently released during hypoxia. Considering that pulmonary hypertension is usually associated with hypoxemia, its pathogenesis may crucially involve a simultaneous change of the inflammatory system.

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