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Pattern of NOS2 and NOS3 mRNA expression in human A549 cells and primary cultured AEC II

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Pechkovsky, D. V., G. Zissel, T. Goldmann, M. Einhaus, C. Taube, H. Magnussen, M. Schlaak, and J. Müller-Quernheim. Pattern of NOS2 and NOS3 mRNA expression in human A549 cells and primary cultured AEC II. *Am J Physiol Lung Cell Mol Physiol* 282: L684–L692, 2002. First published October 26, 2001; 10.1152/ajplung.00320.2000.—The human alveolar type II epithelium-like cell line A549 expresses nitric oxide synthase type 2 (NOS2), but not NOS3, and produces nitric oxide (NO) upon appropriate stimulation. However, relatively little is known regarding the NOS2 and NOS3 expression of type II human alveolar epithelial cells (AEC II) in primary culture. We detected NOS3 mRNA in freshly isolated AEC II and after 24 h of culture. NOS3 mRNA levels were much higher in AEC II cultured for 24 h with or without interferon-γ, interleukin-1β, and tumor necrosis factor-α, compared with freshly isolated cells. Cytokine stimulation did not change the NOS3 mRNA expression level in AEC II compared with unstimulated cells. NOS3 protein expression was verified by Western blot, and measuring nitrate/nitrite revealed that the protein is active. In contrast, neither NOS2 mRNA nor protein could be detected in freshly isolated, unstimulated or cytokine-stimulated human AEC II in 24- or 72-h primary cultures, whereas A549 cells expressed NOS2 message and protein upon stimulation with proinflammatory cytokines. In situ hybridization confirmed that AEC II express NOS3, but not NOS2 mRNA in vivo. These data demonstrate that there are significant differences between primary AEC II and A549 cells in NOS mRNA expression pattern.

nitric oxide synthase; mRNA; RT-PCR; interferon-γ; tumor necrosis factor-α; interleukin-1β; type II human alveolar epithelial cells; A549 cell line

NITRIC OXIDE SYNTHASES (NOS) constitute a family with at least three distinct isoforms; neuronal (nNOS or NOS1), inducible (iNOS or NOS2) and endothelial (eNOS or NOS3) (20, 21). NOS1 and NOS3 are constitutively active and produce small amounts of nitric oxide (NO), whereas NOS2 is induced by inflammatory cytokines and produces a larger amount of NO. All three types of NOS are expressed in human lung, and NOS mRNA expression and enzyme activity have been described in human bronchial epithelial cells, macrophages, endothelial cells, and vascular smooth muscle cells (18, 34). However, relatively little is known regarding the expression of NOS2 and NOS3 in type II human alveolar epithelial cells (AEC II) and how these enzymes are regulated. Although several laboratories have reported that rat AEC II express NOS2 mRNA and produce NO in vitro (12, 26) and that human alveolar epithelium exhibits an NOS-like enzymatic activity in vivo (18), there has been neither molecular nor biochemical confirmation of the expression of NOS2 and NOS3 in human primary cultured AEC II. Asano et al. (4) and Kwon and George (19) have demonstrated that the human type II alveolar epithelial cell line (A549) expresses NOS2 and produces NO in response to proinflammatory cytokines such as interferon-γ (IFN-γ), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α). Therefore, the aims of the present study were *1*) to determine whether human AEC II express NOS2 and NOS3 mRNA in primary culture, *2*) to characterize the regulation of mRNA expression of NOS isoforms in these cells by proinflammatory cytokines, and *3*) to compare these results with data from the A549 cell line. We report here that NOS2 mRNA expression and protein accumulation are absent in unstimulated and cytokine-stimulated human primary AEC II that were isolated with high purity. In contrast, NOS3 mRNA and protein are present in freshly isolated or cultured cells, and NOS3 mRNA expression can be modulated in vitro. Additionally, we show that the human lung adenocarcinoma cell line A549, which is commonly used to study human AEC II properties, has a different pattern of NOS isoforms expression compared with human primary AEC II.

MATERIALS AND METHODS

Reagents. The following materials were purchased from GIBCO-BRL (Paisley, UK): dispase, PBS, RPMI 1640 medium, FCS, HEPES, TRIzol reagent, SuperScript RNase H$^-$ RT, oligo(dT)$_{12–18}$ primer, and agarose. Ficoll separation isotonic solution (density 1.077), penicillin/streptomycin solution, and sodium pyruvate solution were from Biochrom (Berlin, Germany); trypsin-EDTA solution and DNase I were

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from Boehringer-Mannheim (Mannheim, Germany); collagen R was from Serva (Heidelberg, Germany); chloroform and isopropanol were from Merck (Darmstadt, Germany); recombinant human IFN-γ (specific activity 3×10^7 U/mg) and recombinant human IL-1β (specific activity 2×10^8 U/mg) were from Biotrend (Köln, Germany); nylon gauze was from Eckert (Waldkirch, Germany); 100-mm plastic dishes, 75-cm^2 tissue culture flasks, and 24-well cell culture plates were from NUNC (Wiesbaden, Germany). Lipopolysaccharide (LPS, *Salmonella minnesota*) was kindly provided by Dr. K. Brandenburg (Research Centre Borstel, Borstel, Germany), and recombinant human TNF-α was courtesy of Dr. E. Schlick (Knoll, Ludwigshafen, Germany).

Culture of A549 cells. A549 cells were a generous gift of Dr. T. Papadopoulos (University of Erlangen, Erlangen, Germany). Experiments were conducted with freshly thawed cells and cells after 2, 4, 6, and 7 passages. Cells were grown on 75-cm^2 tissue culture flasks in culture medium (CM) (RPMI 1640 medium with 2 mM L-glutamine, 10% heat-inactivated FCS, 1% penicillin-streptomycin solution, 1% sodium pyruvate solution, and 20 mM HEPES) in a humidified atmosphere containing 5% CO_2 at 37°C for 5 days. After this culture period, cells were removed from the plastic surface by treatment with trypsin-EDTA solution (0.05/0.02% in PBS) for 10 min at 37°C, washed twice in PBS, and resuspended in CM.

Human lung tissue. Lung tissue samples were obtained from subjects with central lung cancer undergoing lobectomy or pneumectomy. Five patients with bronchogenic carcinoma, proven histologically before treatment, without other systemic diseases were enrolled in this study. All subjects were smokers and had no respiratory tract infection within the last month. None of them was taking antibiotics or immunosupressants at the time of operation. Informed consent was obtained from each subject. The study was approved by the medical ethics committees of the involved institutions.

Isolation and culture of human AEC II. Samples from macroscopically tumor-free lung tissue were cut from the surgical specimens. The lung tissue was first sliced, and slices were washed three times at 4°C in PBS. The washed slices were incubated in sterile dispase solution at 37°C for 45 min. After dispase digestion, the lung tissue slices were cut into small, pipettable pieces and thoroughly pipetted for 10 min. Crude tissue and cell suspensions were filtered through nylon gauze with meshes of 100, 50, and 20 μm. The resulting single-cell suspension was placed on Ficoll separating solution and centrifuged at 800 *g* for 20 min. The AEC II-enriched cells from the interphase were incubated in 100-mm plastic dishes at 37°C in humidified air containing 5% CO_2 for 15, 20, and 30 min with seeding of nonadherent cells on fresh dishes for each time interval to remove adherent cells (alveolar macrophages, monocytes, fibroblasts, and endothelial cells). To remove remaining monocytes/macrophages and lymphocytes, antibodies against CD3 (OKT3, ECACC 86022706) and CD14 (ATCC HB-246) were added, and the antibody-binding cells were removed by anti-mouse IgG-coated magnetic beads and a magnetic-activated cell sorting (MACS) system (Miltenyi Biotec, Bergisch Gladbach, Germany) as suggested by the supplier. The type II alveolar epithelial nature of the isolated cells was confirmed by modified Papanicolaou staining (16), alkaline phosphatase activity assay (8), and surfactant protein A (SP-A) mRNA expression in RT-PCR. Cell purity was assessed by immunoperoxidase staining with monoclonal antibodies directed against CD3 and CD14 (Immunotech, Marseille, France) as described earlier (37). Viability of the AEC II after isolation was >97% as determined by trypan blue exclusion.

Stimulation with cytokines. To assess the effect of stimulation on NOS gene expression, AEC II were treated with CM, LPS (1 μg/ml), TNF-α (1–10 ng/ml), IFN-γ (10–100 U/ml), or IL-1β (10–100 U/ml) and with a combination of these stimuli in collagen R-coated 24-well plates (1×10^6 cells/ml; 1 ml/well) in a humidified atmosphere containing 5% CO_2 at 37°C for 24 h. For time course experiments, cells were cultured without stimulus, with LPS, or with one of the following cytokines: TNF-α, IFN-γ, or IL-1β in the concentration given above. Cells were harvested after 4, 8, 16, 24, 48, or 72 h of culture. A549 were treated with CM alone, LPS (1 μg/ml), or cytokines for 4, 8, or 24 h using the same protocol as for primary AEC II. After culture the cell viability always exceeded 95% in both AEC II and A549 as determined by trypan blue exclusion. Cell culture supernatants were removed and cells were lysed with TRIzol reagent. Cell lysates were frozen and kept at −70°C before the RNA isolation procedure.

RT-PCR. Total RNA was extracted from cells using TRIzol according to the manufacturer's protocol (GIBCO-BRL). Equal amounts of total RNA from each sample were primed with oligo(dT) and reverse transcribed with SuperScript RT for 1 h at 37°C to produce cDNA. PCR amplification of the cDNA (2.5 μl) from the reverse transcription reaction was carried out using specific primer pairs for β-actin, NOS2, and NOS3. Sequences of the primers for analysis of mRNAs were NOS2 (forward): TCC GAG GCA AAC AGC ACA TTC; NOS2 (reverse): GGG TTG GGG GTG TGG TGA TGT; NOS3 (forward): GTG ATG GCG AAG CGA GTG AAG; NOS3 (reverse): CCG AGC CCG AAC ACA CAG AAC; β-actin (forward): AGC GGG AAA TCG TGC GTG; β-actin (reverse): CAG GGT ACA TGG TGG TGCC. All primers were synthesized by MWG-Biotech (Ebersberg, Germany). Target cDNA was amplified using a three-temperature PCR and an automated thermocycler (Biometra, Göttingen, Germany) according to Reiling et al. (29) with primer pairs for NOS2 and NOS3. PCR conditions for β-actin amplification included: 95°C for 1 min, 57°C for 1 min, and 72°C for 1 min 30 s. The numbers of cycles were the same for NOS2, NOS3, and β-actin (30 cycles). PCR products (length: 462 bp for NOS2, 422 bp for NOS3, 309 bp for β-actin) were electrophoresed on 1.5% agarose gels and stained with ethidium bromide. Gel analysis was done densitometrically with the Gel Doc 2000 gel documentation system and Quantity One 4.0.3 software (Bio-Rad Laboratories, Hercules, CA). To ensure the identity of the PCR-amplified fragments, the size of each amplified mRNA fragment was compared with DNA standards (100 bp DNA Ladder; GIBCO-BRL) electrophoresed on the same gel. Additionally, the NOS2 and NOS3 specificity of the PCR product was verified by comparing their sequence data with the published ones (27). The NOS2- and NOS3-specific primers used in the RT-PCR were intron spanning, and therefore genomic DNA contamination did not influence the PCR results.

Immunocytochemistry. AEC II or A549 cells (2.5×10^5 cells) were plated in duplicate chambers of an eight-chamber glass slide (Flexiperm-Slide, W. C. Heraeus, Hanau, Germany) and cultured in CM, in the absence or presence of IFN-γ (50 U/ml) or a mixture of cytokines [TNF-α (5 ng/ml), IFN-γ (50 U/ml), and IL-1β (50 U/ml)], or LPS (1 μg/ml) for 18 h in a humidified atmosphere containing 5% CO_2 at 37°C. After incubation, slides were washed in PBS, and cells were fixed in 3.7% paraformaldehyde in PBS for 10 min. After fixation, slides were washed three times in PBS with 0.1% saponin. Endogenous peroxidase was blocked by incubation in 0.3% hydrogen peroxide-0.02% sodium azide for 15 min, followed by a further incubation in 10% normal goat serum in

PBS with 0.1% saponin for 10 min. Between the various steps, the slides were washed in PBS-saponin at room temperature. The slides were incubated for 2 h at room temperature with a murine monoclonal anti-human NOS2 antibody (Transduction Laboratories, Lexington, KY) at a concentration of 4 μg/ml in PBS with 0.1% saponin (31). Control slides were incubated with saponin-PBS alone. After being washed and incubated with a biotinylated goat anti-mouse IgG antibody (Jackson ImmunoResearch Labs, West Grove, PA) for 30 min and streptavidin horseradish peroxidase (Zymed Labs, South San Francisco, CA) for 30 min, the slides were treated with diaminobenzidine in PBS, counterstained with hematoxylin, mounted in glycerol gelatin, and coverslipped.

Western blotting analysis for NOS2 and NOS3. AEC II or A549 cells (5×10^6) that had been incubated with or without appropriate stimuli were removed from flasks by treatment with trypsin-EDTA solution for 10 min at 37°C, washed in PBS, resuspended in 0.2 ml of a lysis buffer [50 mM Tris·HCl, pH 7.6, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.1% of Triton X, 1% protease inhibitor cocktail (Sigma, St. Louis, MO)], and lysed for 30 min on ice. The cell debris was removed by centrifugation at 13,000 *g* for 15 min. NOS2 and NOS3 proteins were enriched by adenosine 2′5′-ADP-agarose (Sigma, Deisenhofen, Germany). Aliquots of this enriched fraction containing 25 μg of protein were resolved by SDS-PAGE (10% acrylamide) and transferred onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Little Chalfont, UK) by using a semidry technique (Hoefer Semi-Dry Transfer Unit; Amersham Pharmacia Biotech). The membranes were labeled with monoclonal mouse anti-NOS2 and anti-NOS3 antibodies, as suggested by the supplier (Transduction Laboratories). The blots were developed using the enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) technique according to the manufacturer's instructions.

In situ hybridization. Paraffin-embedded lung tissue samples were prepared from the same surgical specimens as described before and used for in situ hybridization (ISH). These tissue samples showed normal architecture with few intra-alveolar macrophages and edema. The cDNA probes corresponding to the NOS2 and NOS3 genes were produced by PCR as described before, filtered through Centri-Sep spin columns (Applied Biosystems, Foster City, CA), and labeled with dioxigenin (DIG) following the manufacturer's instructions (DIG-High Prime, Roche, Germany). After deparaffinization, ISH was carried out overnight, and, after washing at high stringency, detection was performed by application of anti-DIG/alkaline-phosphatase-conjugate and new fuchsin as substrate for the alkaline phosphatase (9). For negative control, sections were hybridized with the hybridization buffer in the absence of labeled cDNA probes. Hybridization of a probe targeting the RNA of SP-A, a specific product of AEC II, served as an additional positive control.

Nitrate/nitrite determination. Total NO products were determined by a nitrate/nitrite (NO_x) colorimetric assay kit (Cayman Chemical, Ann Arbor, MI) according to the suggestions of the supplier. This test employs a Griess reaction after enzymatic conversion of NO_3^- into NO_2^-. The detection limit of this assay is 2.5 μM.

Statistical analysis. Data are expressed as means ± SE. Statistical comparisons were made by analysis of variance (ANOVA) with post hoc Fisher's protected least significant difference for each agent separately. Probability values of <0.05 were considered significant. All analyses were done using the StatView 4.02 program (Abacus Concepts, Berkeley, CA) for Macintosh computers.

RESULTS

Characteristics of isolated AEC II. In response to the difficulties in demonstrating NOS production and NO release by human cells, we focused our studies on more sensitive methods to identify NOS in human AEC II by analyzing mRNA expression of NOS2 and NOS3 by RT-PCR (1, 28). A critical point of this approach is the purity of the cells, since the high sensitivity of the assay may detect mRNA of contaminating cells. Ex vivo isolation of AEC II is regularly associated with some degree of contamination by lung mononuclear cells that express NOS2 and NOS3 in vitro, especially after cytokine stimulation (26–28). Therefore, in the present study we used human AEC II isolated by three steps of purification with depletion of all possible contaminating cells, as described in MATERIALS AND METHODS. These methods allowed us to purify AEC II to >96%, thus eliminating artifacts, which may be introduced by RNA derived from other lung mononuclear cells. After the final step of MACS purification, five AEC II preparations included in this report were free of $CD14^+$ and $CD3^+$ cells as determined by immunocytochemistry, 97 ± 1.9% of cells were identified as AEC II by the presence of dark blue inclusions revealed by modified Papanicolaou staining, and 94 ± 1.1% of cells were positive for alkaline phosphatase (Fig. 1, *A*

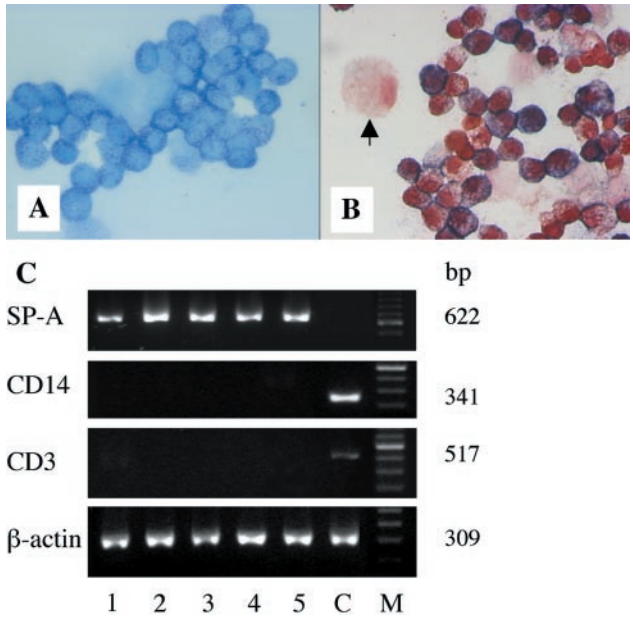

Fig. 1. *A* and *B*: photomicrographs of freshly isolated type II human alveolar epithelial cells (AEC II) stained with modified Papanicolaou stain (*A*), and alkaline phosphatase stain, counterstained with 0.1% neutral red (*B*). Specific inclusion bodies within the cytoplasm are present in all of the cells (*A*), and blue reaction product identifies alkaline phosphatase activity in almost all cells. Alveolar macrophages stain only with the neutral red counterstain (arrow, *B*). *C*: results of RT-PCR product analyses with RNA from 5 freshly isolated AEC II preparations (*lanes 1–5*, respectively) and specific primers for surfactant protein (SPA)-, CD3, CD14, and β-actin, serving as an internal control. *Lane C* shows the results of RT-PCR with RNA isolated from freshly obtained human bronchoalveolar lavage cells used as positive control for CD3 and CD14, and as negative control for SP-A; *lane M* indicates the molecular marker.

and *B*). All RNA samples isolated from these five AEC II preparations contained SP-A mRNA and were free of CD3 and CD14 mRNA as determined by RT-PCR (Fig. 1*C*).

NOS expression in AEC II. PCR for NOS3 yielded a fragment of the expected size from all fresh noncultured AEC II preparations (Fig. 2*A*). After 24 h culture the PCR product appeared as a strong intensive band independent from the presence or absence of proinflammatory cytokines at all concentrations tested. By densitometric analysis, we noted that there were no differences in NOS3 expression in cells with or without cytokine stimulation, but the level of NOS3 mRNA expression in cells cultured without stimulation was significantly higher compared with that of freshly isolated cells (Fig. 2*B*). RT-PCR with RNA samples from freshly isolated, unstimulated, and cytokine-stimulated AEC II did not reveal the presence of any NOS2 mRNA (Fig. 2*A*). NOS2 gene expression could not be detected in cells stimulated with IFN-γ at the concentrations of 10, 50, and 100 U/ml for 4, 8, 16, 24, 48, and

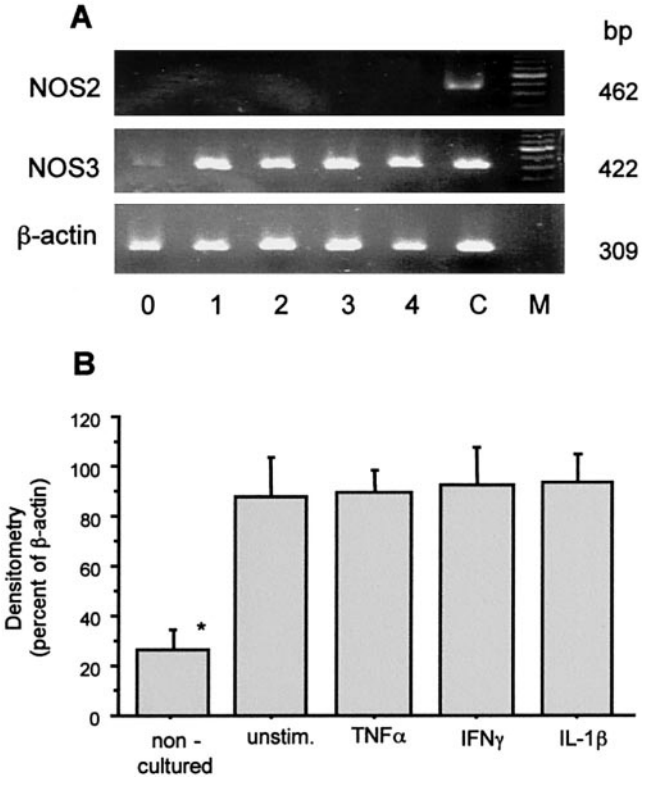

Fig. 2. Nitric oxide synthase type 2 (NOS2) and NOS3 mRNA analysis of noncultured and primary cultured human AEC II by RT-PCR. Cells were cultured without (unstim) or with tumor necrosis factor (TNF)-α (5 ng/ml), interferon (IFN)-γ (50 U/ml) or interleukin (IL)-1β (50 U/ml) for 24 h. *A*: representative images of NOS2 and NOS3 mRNA expression in AEC II derived from 1 of 5 identical experiments. Expression of β-actin in the same samples demonstrates equal loading of lanes. *Lanes 0–4*, cells noncultured, unstimulated, and stimulated with TNF-α, IFN-γ, or IL-1β. *Lane C*, positive control bands; *lane M*, molecular marker. *B*: densitometric analysis of the NOS3 mRNA expression. The mRNA amplificates from each culture were quantitated individually. Values presented are the mean percentages of β-actin density ± SE ($n = 5$). *$P < 0.01$ compared with unstimulated or cytokine-stimulated cells.

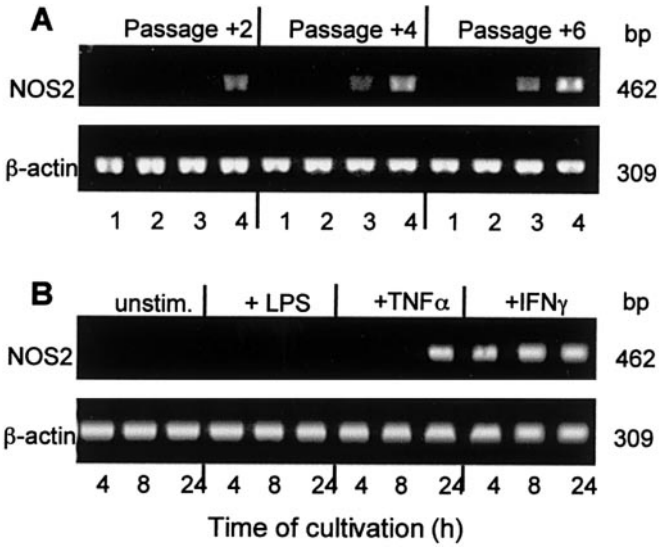

Fig. 3. NOS2 mRNA expression in A549 cells stimulated with IFN-γ (50 U/ml), TNF-α (5 ng/ml), or lipopolysaccharide (LPS, 1 μg/ml) for 24 h and its dependence on the number of cell passages. *Lanes 1–4*, unstimulated cells and LPS-, TNF-α-, and IFN-γ-stimulated, respectively (*A*). Time course experiment with A549 cells after 7 passages, effects of LPS or cytokine stimulation for 4, 8, and 24 h on NOS2 mRNA expression are presented (*B*).

72 h, as well as in those stimulated with LPS (1 μg/ml), TNF-α (1, 5, and 10 ng/ml) or IL-1β (10, 50, and 100 U/ml), and with its combinations at the same periods of time (data not shown). All RNA samples isolated from these cultured AEC II were positive for SP-A, and intensities of the bands were not different in unstimulated and cytokine-stimulated cells during all time periods of culture (data not shown).

NOS expression in A549 cells. Stimulation of A549 cells with IFN-γ or TNF-α for 24 h resulted in an upregulation of NOS2 gene expression. However, the induction of NOS2 mRNA by these cytokines was closely dependent on the numbers of cell culture passages after thawing and inoculation of A549 cells in culture. With an increase in the number of passages, the A549 cells appeared to become more responsive to cytokine stimulation. As shown in Fig. 3*A*, treatment of the A549 cells with 50 U/ml of IFN-γ induced NOS2 mRNA expression in all cultures, and this response was more prominent in cultures established for a longer period of time (*passages 4* and *6*). Presence of TNF-α for 24 h in A549 cell culture after two passages did not lead to induction of NOS2 message, but in cultures established after four or six passages TNF-α at a concentration of 5 ng/ml was able to induce the NOS2 mRNA expression. However, the TNF-α-induced NOS2 mRNA expression levels were relatively low compared with those upon IFN-γ stimulation. In an additional experiment with A549 cells after seven passages, these cells were kept quiescent or stimulated with 1 μg/ml of LPS, 5 ng/ml of TNF-α, or 50 U/ml of IFN-γ for 4, 8, and 24 h. RT-PCR analysis demonstrated NOS2 mRNA at all three time points in IFN-γ-stimulated cells and only at 24 h in TNF-α-stimu-

lated cells, whereas in the other two conditions no NOS2 mRNA could be detected (Fig. 3*B*). Culture of A549 cells with LPS alone at a concentration of 1 μg/ml did not lead to upregulation of the NOS2 expression in any of the experimental conditions tested (Fig. 3, *A* and *B*). In none of the conditions described above could NOS3 mRNA be detected in A549 cells (data not shown).

NOS2 and NOS3 mRNA expression by AEC II in vivo. ISH using DIG-labeled DNA probes detected specific signals for NOS2 and NOS3 mRNA mainly in intra-alveolar macrophages in all lung tissue preparations included in the present study. No positive signals for NOS2 mRNA were detected in alveolar epithelium, including AEC II, which were typically localized at alveolar corners (Fig. 4*A*). In contrast, positive signals for NOS3 mRNA were detected in AEC II, especially in proliferated cells, as well as in resident and free-located alveolar macrophages (AM) (Fig. 4*B*). Nonspecific signals were not detected in control preparations, in which specific DNA probes were substituted by hybridization buffer alone (not shown).

Immunocytochemistry. The differences in NOS2 mRNA expression of A549 cells and AEC II were confirmed by studying protein expression of these cells immunocytochemically. A549 cells and AEC II were stimulated with cytokines for 18 h and immunostained with anti-human NOS2 antibodies as described in MATERIALS AND METHODS. Neither unstimulated AEC II nor AEC II stimulated with IFN-γ or a mixture of cytokines (TNF-α, IFN-γ, and IL-1β) displayed immunoreactivity for NOS2 (Fig. 4*C*), whereas A549 cells expressed immunoreactivity in the presence of IFN-γ (data not shown) or a mixture of cytokines (Fig. 4*E*). Thus whereas unstimulated A549 cells did not exhibit any immunoreactivity, stimulation with IFN-γ or a cytokine mixture caused clear-cut NOS2 protein expression compared with unstimulated cells (Fig. 4*D*). However, not all cells showed unequivocal staining. The positively stained cells were more rounded and less attached to the surface of the culture chamber (Fig. 4*E*). Nonstaining cells showed typical morphology as determined by standard light microscopy (Fig. 4, *D* and E). Iden-

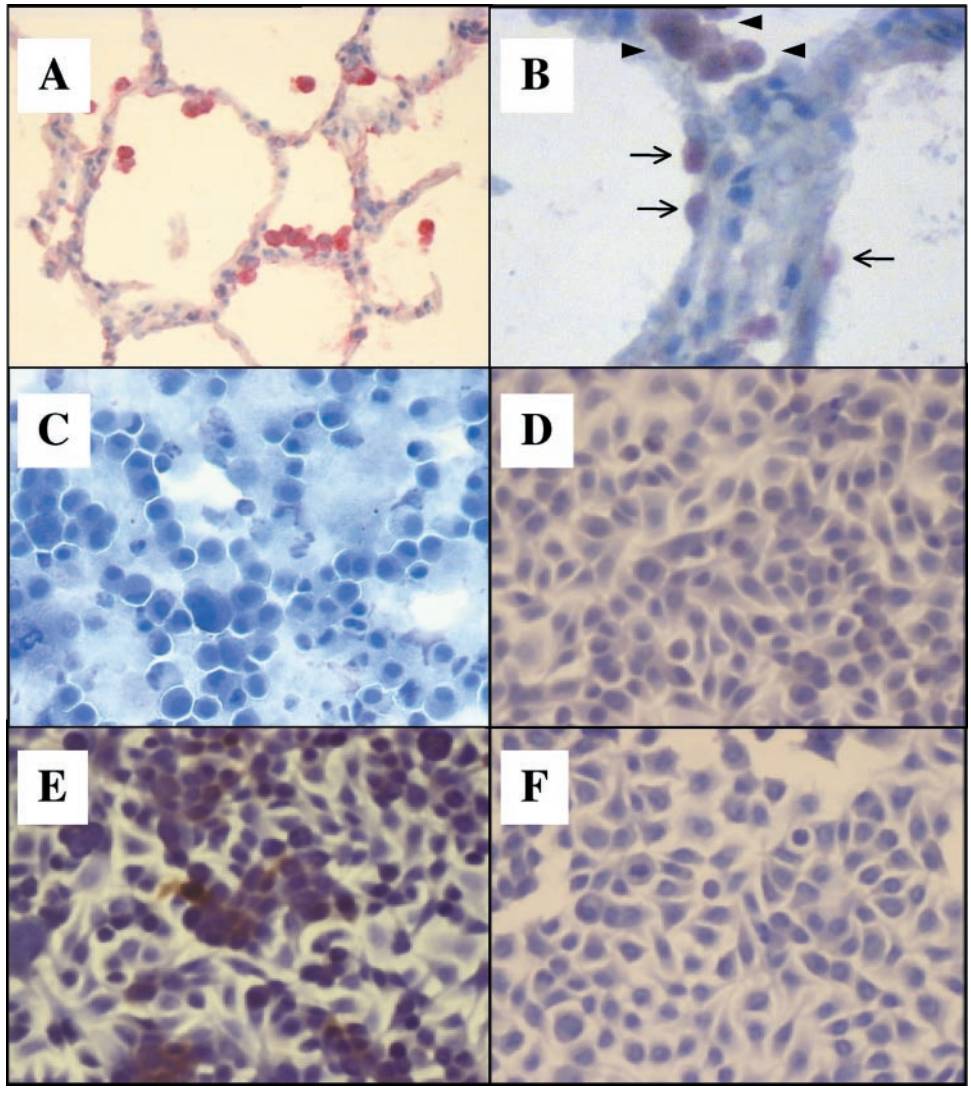

Fig. 4. In situ hybridization for NOS2 and NOS3 mRNA and immunocytochemistry for NOS2 protein. NOS2 mRNA expression is seen only in alveolar macrophages located in the lumen of the alveoli (*A*). *B*: NOS3 mRNA expression is visible in alveolar macrophages (arrowheads) and in AEC II (arrows). *C*: AEC II stimulated with a combination of TNF-α (5 ng/ml), IFN-γ (50 U/ml), and IL-1β (50 U/ml) for 18 h. Similar results were obtained with AEC II unstimulated or stimulated with IFN-γ (50 U/ml) (not shown); A549 cells unstimulated (*D*) and stimulated with a combination of TNF-α (5 ng/ml), IFN-γ (50 U/ml), and IL-1β (50 U/ml) for 18 h (*E*); the same cells stained without primary antibodies (*F*, negative control).

tical results were obtained with all AEC II preparations involved in the present study.

Western blot. Cell lysates from highly purified AEC II stimulated with proinflammatory cytokines or their combinations for 24 h were fractionated on SDS-PAGE gels and examined for the presence of NOS2 and NOS3 proteins by Western blot analysis. Evaluating crude cell lysates revealed neither NOS2 nor NOS3 protein. After enrichment with 2′5′-ADP-agarose, NOS3 protein was detectable in both unstimulated and IFN-γ stimulated AEC II (Fig. 5*A*); however, no NOS2 protein was observable.

NO_x concentration. Examination of cell culture supernatants from unstimulated highly purified AEC II revealed an NO_x concentration of 13.3 ± 3.7 nmol·ml^{-1}·10^{-6} cells·24 h^{-1} (Fig. 5*B*). Stimulation with proinflammatory cytokines did not change NO_x generation by AEC II (IFN-γ: 15.6 ± 2.3 nmol·ml^{-1}·10^{-6} cells·24 h^{-1}, TNF-α: 14.5 ± 4.2 nmol·ml^{-1}·10^{-6} cells·24 h^{-1}, IL-1β: 14.5 ± 3.5 nmol·ml^{-1}·10^{-6} cells·24 h^{-1}). In the cell-culture supernatants of unstimulated A549, a mean NO_x concentration of 35.9 ± 23.2 nmol·ml^{-1}·10^{-6} cells·24 h^{-1} could be detected. Again, stimulation with proinflammatory cytokines did not change NO_x generation (IFN-γ: 38.9 ± 22.5 nmol·ml^{-1}·10^{-6} cells·24 h^{-1}, TNF-α: 39.0 ± 22.5 nmol·ml^{-1}·10^{-6} cells·24 h^{-1}; data not shown).

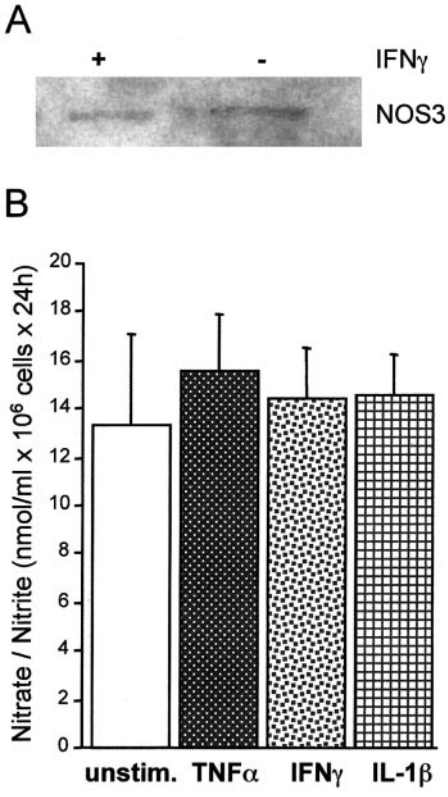

Fig. 5. *A*: Western blot analysis of NOS3 protein in AEC II. Baseline NOS3 expression of cultured nonstimulated AEC II (−) was not altered by the stimulation with IFN-γ. *B*: nitrate/nitrite (NO_x) content in culture supernatants of AEC II either unstimulated or stimulated with cytokines as indicated. None of the cytokines tested induced a significant increase in the NO_x generation of AEC II.

DISCUSSION

In this study, we demonstrated that NOS3 is the predominant NOS mRNA to be found in AEC II isolated from noninvolved lung tissue of patients with central bronchial carcinoma. In contrast, neither NOS2 mRNA nor protein could be detected in these cells ex vivo or after cytokine stimulation in vitro. In contrast to freshly isolated and primary cultured human AEC II, A549 cells expressed NOS2 mRNA after IFN-γ and TNF-α stimulation and cell responsiveness to cytokine stimulation increased with an increasing number of passages after thawing. In accordance with others (4, 19) we have demonstrated that A549 cells are very sensitive to IFN-γ stimulation in their capabilities to express NOS2 and generate NO (4, 19). It has been shown that NO is a strong trigger for growth arrest during cell differentiation, and induction of NOS2 by IFN-γ is associated with this process in immortalized epithelial cells (2). Possibly, this growth inhibitory pathway associated with NOS2 and IFN-γ is also involved in differentiation and growth regulation of permanently cultured A549 cells. Further indirect evidence for a role of NOS2-derived NO as a noninflammatory mediator stems from immunocytochemical study of protein localization in cytokine-stimulated cells. The number of positively stained cells was relatively low, and these cells have had nontypical morphology of cultured A549 cells displaying diminished capability of adherence. No differences are detected in NO_x generation by unstimulated and cytokine-stimulated A549 cells, which in turn, may reflect the low content of NOS2-producing cells. These additional findings suggest, but do not prove, that cytokine-induced NOS2 expression in A549 cells may be connected with differentiation and growth regulation processes rather than with inflammatory mechanisms. We found no evidence of NOS3 expression in unstimulated or cytokine-stimulated A549 cells. These findings are in line with other observations of NOS expression in cell lines (35). In the study by Asano and associates (4), eNOS, nNOS, and iNOS expression was examined by RT-PCR in A549 and BEAS-2B cell lines, and no evidence of NOS3 expression was found in these cell lines. However, NOS2 mRNA was detected in cytokine-stimulated cells in abundant amounts.

Our study raises the question as to the most suitable cell culture model for the study of NO biology in the human alveolar epithelium. A549 cells are a well-established carcinomatous alveolar epithelial cell line and used in various investigations as a model for in vitro characterization of human alveolar epithelial cell functions. Our findings demonstrate that this cell line exhibits striking differences in NOS expression compared with human primary AEC II. It has been demonstrated that rat AEC II express NOS2 and produce significant quantities of NO in response to inflammatory cytokines; this production was comparable with that observed in rat alveolar macrophages (12, 26, 34). Our data indicate that AEC II isolated with high purity from normal lung tissue of cancer patients do not

express detectable NOS2 message or protein ex vivo. In vitro stimulation of these cells with proinflammatory cytokines did not alter the expression of NOS2 mRNA or protein nor did it increase NO_x production by cultured cells. This is in contrast to other published studies, where increased NOS2 expression and NO production were shown in other types of human and rodent airway epithelial cells and immortalized epithelial cell lines after stimulation (4, 11, 26, 30, 34, 35). Possible explanations for this discrepancy include species-specific differences in NOS2 expression or a different physiological/pathophysiological role of this enzyme in rodents and humans (15). It may also be possible that the stimuli and culture conditions that we used are inadequate to stimulate human AEC II to express NOS2 mRNA and, therefore, do not represent the complex dynamic processes occurring in vivo, especially during lung inflammation. Although several reports concerning NOS2 expression in normal human lung tissue have provided evidence that peripheral lung parenchyma, as well as resident lung macrophages, do not express NOS2 mRNA (10) and that no specific immunostaining for NOS2 protein can be observed in alveolar compartments (18), the accumulation of NOS2 protein and NO production have been documented in AM isolated from patients with active pulmonary tuberculosis (22) and acute respiratory distress syndrome (17). However, many attempts to induce NOS2 mRNA expression and/or NO production by human AM in vitro failed (5, 7, 10). In contrast to data from Guo et al. (10) and Kobzik et al. (18), but in accordance with our findings, Saleh et al. (31) demonstrated a weak expression of nitrotyrosine and NOS2 in the airway epithelium and AM and abundant expression of NOS3 in the pulmonary endothelium and airway epithelium of normal parts of human lungs obtained by pneumectomy performed because of lung cancer. In contrast, in lungs of patients with idiopathic pulmonary fibrosis, strong expression of nitrotyrosine, NOS2, and NOS3 was seen in inflammatory cells, AM, and airway epithelium (31). Thus our "in vitro" findings corroborate well results of NOS expression studies "in vivo." Indeed, we were able to show that human AEC II express NOS3 mRNA, in contrast to NOS2, in vivo and ex vivo, and its expression can be upregulated in vitro. In pulmonary endothelial cells, monocyte-derived secretory products or adhesion of monocytes to endothelial cells downregulates steady-state levels of NOS3 due to a shortening of its mRNA half-life time (36). One could speculate that TNF-α or other macrophage-derived products might be responsible for the modulation of NOS3 expression in AEC II. However, we could not identify any effects of exogenously added proinflammatory cytokines (IFN-γ, TNF-α, IL-1β) on NOS3 mRNA expression in AEC II. Additionally, the release of NO_x was not increased by stimulation with proinflammatory cytokines. At the same time, the levels of NOS3 mRNA expression in cells cultured without stimulation were significantly higher compared with that of freshly isolated cells. The exact mechanism by which NOS3 message is upregulated in primary cultured AEC II is unclear. It has been shown that NOS3 mRNA is increased in proliferating bovine aortic endothelial cells (BAEC) as a result of increased mRNA stability (3). In addition, transforming growth factor-β1 (TGF-β1) induced a modest upregulation of NOS3 mRNA in BAEC on the transcriptional level (14). It is possible that the abovementioned factors may also be involved in upregulation of NOS3 mRNA in human AEC II, e.g., high TGF-β production by primary cultures of AEC II as described earlier by our group (37). Which molecular mechanisms regulate NOS3 expression in human AEC II is still not established. In contrast to NOS3, NOS2 is inhibited by TGF-β, both on the transcriptional and translational levels. Additionally, TGF-β downregulates NO_x production of NOS2 (reviewed in Ref. 33). AEC II and A549 express high levels of TGF-β mRNA and release large amounts of TGF-β protein into the cell culture supernatants. This might explain why we could not demonstrate NOS2 protein or an increase in NO_x production in cytokine-stimulated A549 although they express a detectable amount of NOS2 mRNA.

In comparison with NOS3, NO production by NOS2 is higher and predominantly contributes to inflammatory responses induced by endotoxin, cytokines, or physicochemical stress. It is likely that NOS2 expression in human alveolar epithelium is under strong control and that the NOS2 gene is more difficult to induce than in rodents or in human immortalized cell lines. These restrictions in NOS2 expression may have an important physiological basis. Excessive production of NO by NOS2 for long periods of time may be cytostatic or even cytotoxic for pulmonary cells because of the formation of nitrous oxide or peroxynitrite radicals. Furthermore, release of NO from cells in the alveolar compartment may be harmful to the organism, since gas exchange could be reduced due to a reduction of the oxygen-carrying capacity of hemoglobin, or pulmonary vessel tone could be lowered, because of the close proximity of AEC II and the lung vascular compartment. However, recent findings illustrate that "inducible" NOS2 is expressed constitutively, for instance, in tracheal and bronchial epithelium of normal human airway, implying a homeostatic role not previously considered for this isoform (10, 11). On the other hand, constitutive NOS isoforms, such as NOS3, produce small amounts of NO, which serves as an intracellular mediator. Interestingly, levels of transcripts for "constitutive" NOS3 can be changed by hyperoxia or hypoxia, by cytokines, or during cell differentiation in lung endothelium and epithelium (23, 24, 36). Moreover, Bucher et al. (6) found an upregulation of NOS3 mRNA expression in the liver of LPS- or lipoteichoic acid-treated rats. They concluded that NOS3 might be an even more important source of NO than NOS2 in the liver after stimulation with LPS (6). In addition to the constitutive expression of NOS3 mRNA in AEC II, we could determine a low level of NOS3 protein in cell lysates from unstimulated and cytokine-stimulated AEC II by Western blotting assay. In separate experiments that exploit the effect of the NOS inhibitor nitro-L-arginine methyl ester (L-NAME) on IL-8 release

by AEC II, we showed that 10^{-4} M L-NAME significantly decreased the spontaneous and cytokine-induced IL-8 mRNA expression and protein production in primary cultured AEC II (25, and unpublished data). These observations are supported by the studies of Villarete and Remick (32) and Inoue et al. (13), in which blockade of endogenously generated NO significantly downregulated IL-8 release in human endothelial and transformed bronchial epithelial cells in vitro. In line with the constitutive expression of NOS3 mRNA and protein in cultured AEC II, no differences in NO_x production could be detected. Thus this study demonstrates that AEC II express functional NOS3, but not NOS2, and produce NO, playing an important role in the regulation of cellular functions in the lower respiratory tract. In addition, our data give strong evidence that NOS regulation by proinflammatory cytokines in human primary AEC II in vitro significantly differs from other types of human airway epithelium or immortalized epithelial cell lines.

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