Nitric oxide inhibits lipopolysaccharide-induced apoptosis in pulmonary artery endothelial cells

GARY D. CENEVIVA,1 EDITH TZENG,2 DALE G. HOYT,3 EMILY YEE,4 ALICIA GALLAGHER, J JOHN F. ENGELHARDT,5 YOUNG-MYEONG KIM,2,3 TIMOTHY R. BILLIAR,2 SIMON A. WATKINS,6 AND BRUCE R. PITT1,3

1Department of Pharmacology; 2Division of Pediatric Critical Care Medicine, Department of Anesthesiology; 3Division of Pulmonary, Allergy, and Critical Care Medicine, and 6Department of Cell Biology and Physiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261; and 5Department of Anatomy and Cell Biology, University of Iowa Medical Center, Iowa City, Iowa 52242

Ceneviva, Gary D., Edith Tzeng, Dale G. Hoyt, Emily Yee, Alicia Gallagher, John F. Engelhardt, Young-Myeong Kim, Timothy R. Billiar, Simon A. Watkins, and Bruce R. Pitt. Nitric oxide inhibits lipopolysaccharide-induced apoptosis in pulmonary artery endothelial cells. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L717–L728, 1998.—Our group recently reported that cultured sheep pulmonary artery endothelial cells (SPAECs) became resistant to lipopolysaccharide (LPS)-induced apoptosis several days after constitutive synthesis of nitric oxide (NO) after adenoviral (Ad) transfer of inducible NO synthase (iNOS) or exposure to the NO donor S-nitroso-N-acetylpenicillamine (SNAP) (E. Tzeng, Y.-M. Kim, B. R. Pitt, A. Lizonova, I. Kovesdi, and T. R. Billiar. Surgery 122: 255–263, 1997). In the present study, we confirmed this observation by establishing stable transfecants after retroviral gene transfer [replication-deficient retrovirus (DFG)] of human iNOS (DFG-iNOS) SPAECs and then used all three approaches (Ad, DFG, and SNAP) to determine underlying mechanisms of this phenomenon. Continuous endogenous production of NO in itself did not cause apoptosis as assessed by phase-contrast microscopy, nuclear morphology, and internucleosomal DNA fragmentation. Prolonged (72–96 h) synthesis of NO, however, after DFG- or replication-deficient adenovirus (Ad.CMV)-iNOS or SNAP (100 µM, 96 h) inhibited LPS-induced apoptosis. The kinetics of such protection suggested that NO may be inducing other gene products. Ad-mediated transfer of manganese superoxide dismutase (MnSOD) decreased the sensitivity of wild-type SPAECs to LPS-induced apoptosis. MnSOD, however, was not induced in an N6-monomethyl-L-arginine (L-NMMA)-sensitive time-dependent fashion after Ad.CMV-iNOS. Other inducible genes that may be affected by NO and that may protect against potential oxidant-mediated LPS-induced apoptosis including 70-kDa heat shock protein, heme oxygenase-1, metallothionein, and Bcl-2 also were not elevated in an L-NMMA-sensitive, time-dependent fashion. Although the candidate gene product underlying NO-induced protection remains unclear, we did note that prolonged synthesis of NO inhibited LPS-induced activation of an interleukin-1β-converting enzyme-like cysteine protease (cysteine protease protein-32-like) in a dithiothreitol-sensitive fashion, suggesting that S-nitrosylation of an important downstream target of convergence of apoptotic signals may contribute to the sensitivity of SPAECs to LPS.

Cysteine protease protein-32-like protease; manganese superoxide dismutase; mitochondria; heme oxygenase; Bcl-2

Apoptosis remains a poorly understood but potentially important process in lung pathology. Since the original report (33) that apoptosis may be a component of repair in chronic interstitial lung disease, investigators (16) have suggested a role for programmed cell death in the response of pulmonary resident and migratory cells during acute lung injury. The factors contributing to apoptosis during acute lung injury and the cellular and molecular mechanisms affecting this process are not well defined.

Nitric oxide (NO) is an important effector molecule in acute lung injury, with both cytotoxic and protective effects (8). The extent of the role of NO is likely to include aspects of apoptosis in the lung. In nonpulmonary cells, high levels of NO promote apoptosis (7, 36), whereas it is becoming more apparent that endogenous NO synthesis or exposure to low levels of NO donors is associated with inhibition of apoptosis (2, 4, 6, 9, 18, 24). The antiapoptotic actions of NO have been ascribed to cGMP-dependent (2, 4, 9, 32) and cGMP-independent (24) pathways or induction of cytoprotective stress genes such as 70-kDa heat shock protein (HSP70) (17). cGMP-dependent pathways in NO-induced inhibition of apoptosis involve upregulation of Bcl-2 (9) and inhibition of caspase-3-like [cysteine protease protein-32 (CPP32)] activity, a critical cysteine protease involved in initiation and amplification of the cell death signaling cascade. Furthermore, CPP32-like proteases may be directly inhibited via protein S-nitrosylation (19, 26). Tzeng et al. (37) recently extended observations regarding NO as an antiapoptotic signaling molecule to the case of pulmonary endothelium and noted that either endogenous or chemically generated NO inhibits lipopolysaccharide (LPS)-induced apoptosis in cultured sheep pulmonary artery endothelial cells (SPAECs). A unique feature of this study was that resistance to LPS-induced apoptosis was time dependent and was not apparent until 96 h after exposure.

In the present study, we continued pursuing mechanisms by which NO is antiapoptotic in pulmonary endothelial cells. We focused on LPS-induced apoptosis in SPAECs because our group has shown that 1) LPS results in a complete spectrum of apoptotic changes in SPAECs (14) that may not be apparent in other species (13); 2) LPS causes synthesis of luciferase enhanced chemiluminescence in SPAECs that is blocked by over-expression of HSP70 (43); and 3) LPS-mediated apoptosis is sensitive to N-acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO), a caspase-3-specific inhibitor (37).
The time-dependent nature of NO-induced inhibition of LPS-mediated apoptosis suggested that NO may be affecting the levels of other protective genes. We chose to examine the interaction between NO and other antioxidants including 1) manganese superoxide dismutase (MnSOD), the expression of which is affected by LPS (29) or NO (15, 23) and the role of which in mediated apoptosis in cultured hepatocytes (18) or LPS-mediated apoptosis in SPAECs (43); 2) HSP70, which our group has shown is upregulated by NO and inhibits tumor necrosis factor (TNF)-α-mediated apoptosis in cultured hepatocytes (18) or LPS-mediated apoptosis in SPAECs (43); 3) heme oxygenase-1 (HO-1), which our group has shown is induced by large amounts of NO from S-nitroso-N-acetylpenicillamine (SNAP) in SPAECs (45) and which contributes to NO-induced inhibition of sensitivity of endothelial cells (30) and hepatocytes (17) to subsequent oxidant or nitrosative stress; 4) metallothionein (MT), which our group has shown reduces the sensitivity of SPAECs to various forms of oxidant injury (31) and is antiapoptotic in mouse embryonic cells (22) and SPAECs (34); and 5) Bcl-2, which is a ubiquitous inhibitor of apoptosis, perhaps secondary to potential antioxidant activity (12). Because the previous study by Tzeng et al. (37) utilized a protocol in which only a subpopulation of SPAECs (~20%) expressed inducible NO synthase (iNOS) in a transient fashion after adenoviral-mediated gene transfer, our first objective was to confirm these results in a simpler system in which 100% of SPAECs produced constant amounts of NO after retroviral-mediated stable integration of iNOS and a selectable marker (neomycin resistance). After noting remarkable similarity in the 72- to 96-h time delay in inhibition by NO of LPS-induced apoptosis in SPAECs after retroviral infection, we used all three systems (retroviral, adenoviral, and chemical donors) to examine the contributing role of the above molecules (MnSOD, HSP70, HO-1, MT, and Bcl-2). Under conditions of these experiments, we found no conclusive evidence that iNOS-derived NO (or chemically generated NO) increased the synthesis of MnSOD, HSP70, HO-1, MT, or Bcl-2 in SPAECs, underscoring our uncertainty of the target-protective gene responsible for the NO- and time-dependent inhibition of apoptosis in SPAECs. Prolonged synthesis of NO, however, reduced the LPS-induced activation of caspase-3, an interleukin-1β-converting enzyme (ICE)-like cysteine protease, that appears critical for LPS-induced apoptosis in SPAECs.

**METHODS AND MATERIALS**

**Isolation and Culture of SPAECs**

Sheep pulmonary arteries were obtained from a nearby slaughterhouse, and endothelial cells were harvested by collagenase type II (0.1% Sigma, St. Louis, MO) digestion (37°C, 30 min) and cultured in Opti-Mem (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), endothelial cell growth supplement (15 µg/ml; Collaborative Biomedical Products, Bedford, MA), 10 U/ml of heparin sulfate (Fisher Scientific, Pittsburgh, PA), 100 U/ml of penicillin (GIBCO), and 100 µg/ml of streptomycin (GIBCO) at 37°C in an atmosphere of 5% CO2. Cells were routinely passaged 1:4 by detachment with a balanced salt solution containing EDTA-trypsin (GIBCO). At the second passage, cells were incubated with 1,1-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate-labeled acetylated low-density lipoprotein (DiL-Ac-LDL; Biomedical Technologies, Stoughton, MA). Cells preferentially incorporating DiL-Ac-LDL were obtained by fluorescent-activated cell sorting (FAC-star, Becton Dickinson Immunocytometry System, San Jose, CA) as previously described (14). Subpassages of cells were routinely homogeneously positive for Dil-Ac-LDL and factor VIII antigen. SPAECs from passage 4 were used for infection.

**Infection of SPAECs with Human iNOS or MnSOD**

SPAECs were infected with either a replication-deficient recombinant retrovirus (DFG; Moloney murine leukemia virus backbone; 38) or adenovirus (Ad.CMV; 37) containing the human hepatocyte iNOS gene (DFG-iNOS and Ad.CMV-iNOS, respectively). The DFG-iNOS vector also contained a neomycin resistance gene, permitting selection with 750 µg/ml of geneticin (G-418; GIBCO). The transfectants (DFG-iNOS or Ad.CMV-iNOS SPAECs) were maintained in complete medium (see above) containing 1 mM Nω-nitroso-N-acetylpenicillamine (SNAP) or L-arginine (L-NMMA), an NOS inhibitor, until 24–96 h before LPS exposure. In some experiments, wild-type SPAECs were exposed to Ad.CMV containing cDNA for human MnSOD (Ad.CMV-MnSOD). These cells (Ad.CMV-MnSOD SPAECs) were studied 48–72 h later for their sensitivity to LPS. As a comparison, SPAECs were infected with Ad.CMV containing either human copper-zinc SOD (Cu,ZnSOD) or β-galactosidase (LacZ) cDNA (Ad.CMV-Cu,ZnSOD and Ad.CMV-LacZ, respectively). Adenoviral gene transfer was accomplished by using a multiplicity of infection of 100 in the presence of polybrene (4 µg/ml), resulting in ~70% efficiency of gene transfer (1).

**Northern Blot Analysis**

Total cellular RNA was isolated from wild-type or transfected SPAECs with RNAzol B as previously described (3). Aliquots containing 20 µg of RNA were electrophoresed on a 0.9% agarose gel, blotted transferred to GeneScreen membranes (NEN, Cambridge, MA), and ultraviolet (UV) auto-cross-linked (UV Stratalinker 1800, Stratagene, La Jolla, CA). The membranes were hybridized overnight at 42°C with cDNA probes to either human hepatocyte iNOS, mouse MT-I, rat MnSOD, human HO-1, or human 18S rRNA. DNA probes (2–4 x 106 counts·min⁻¹·µg⁻¹) were labeled with [32P]dCTP (specific activity 3,000 Ci/mM; NEN) by random priming (Boehringer Mannheim, Indianapolis, IN). The hybridized membranes were serially washed at 53°C with 2× saline-sodium citrate-0.1% SDS-25 mM NaHPO4·1 mM EDTA and 25 mM NaH2PO4·1 mM EDTA-1% SDS solutions. After the membranes were washed, autoradiography was performed by exposure to Kodak X-Omat film at ~70°C in the presence of intensifying screens or directly on filters for phosphorimage analysis.

**Western Blot Analysis**

Wild-type or transfected SPAECs were washed and then resuspended in protease inhibitor buffer [20 mM TES, pH 7.4, 2 mM dithiothreitol (DTT), 10% glycerol, 25 µg/ml of antipain, 25 µg/ml of aprotinin, 25 µg/ml of leupeptin, 25 µg/ml of chymostatin, 50 mM phenanthrene, and 10 µg/ml of pepstatin A] supplemented with 10 µM flavin mononucleotide, 10 µM FAD, and 5 µM tetrahydrobiopterin. The cells were lysed by three freeze-thaw cycles. Protein concentrations were
determined with the bicinchoninic acid protein assay (Pierce, Rockford, IL). Cell lysates (100 µg protein/lane) were electrophoresed on an 8% SDS-polyacrylamide gel. Eluted proteins were then transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The membrane was blocked with 5% nonfat dried milk, phosphate-buffered saline, and 0.1% Tween 20 and then hybridized with either 1) a monoclonal anti-mouse macrophage iNOS antibody (1:2,000 dilution; Transduction Laboratories, Lexington, KY); 2) murine monoclonal IgG (Sigma) antibody to HSP70 at a 1:2,500 dilution, with the secondary antibody a peroxidase-conjugated, goat anti-mouse IgG (Sigma); 3) polyclonal antibody to Bcl-2; or 4) anti-rat MnSOD antiserum (11). After the membrane was washed three times in phosphate-buffered saline containing Tween 20 and nonfat dried milk, a secondary antibody, namely peroxidase-conjugated goat anti-mouse IgG (Schleicher & Schuell), was applied. The membrane was then incubated in enhanced chemiluminescence reagents (NEN) and exposed to Kodak X-Omat film for 1–20 min at room temperature.

Quantification of MnSOD Activity

SOD activity was measured spectrophotometrically (25) in Ad.CMV-MnSOD or Ad.CMV-Cu,ZnSOD SPAECs as well as in DFG-iNOS SPAECs (with or without L-NMMA) before exposure to LPS.

Measurement of Caspase-3-Like Activity

Adherent and nonadherent cells were collected by scraping and resuspended in 100 mM HEPES (pH 7.4), 140 mM NaCl, and protease inhibitors (aprotinin, pepstatin, leupeptin, and phenylmethylsulfonfyl fluoride). The cytosol was isolated by three cycles of freezing and thawing, and a crude cytosolic fraction was obtained as the supernatant from centrifugation at 12,000 g for 20 min at 4°C. An aliquot was incubated with 400 µM N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA; synthetic substrate from Bachem) in cytosol suspension buffer (and 20% glycerol). Caspase-3-like activity was determined colorimetrically at 405 nm after a 1-h incubation at 37°C. In some instances, we preincubated the lysates in 20 mM DTT, an agent known to remove thiol-bound NO groups and are expressed as a fraction of the total cells counted.

Internucleosomal DNA Fragmentation. After LPS treatment of either DFG-iNOS or Ad.CMV-iNOS SPAECs, detached and attached cells were harvested with trypsin and combined. Cells were lysed in 50 mM Tris (pH 8)-10 mM EDTA-0.5% sarkosyl with 50 µg of proteinase K (Boehringer Mannheim) and incubated at 50°C for a minimum of 1 h. Subsequently, 100 µg of DNase-free RNAse A (Boehringer Mannheim) were added to the lysate and incubated at 37°C for an additional hour. DNA was extracted and purified with Tris-buffer-saturated phenol (pH > 7.5), phenol-chloroform-isooamy alcohol (25:24:1), and chloroform-isooamy alcohol (24:1) (GIBCO BRL). DNA was precipitated in 0.3 M sodium acetate (pH 5.2) and ethanol (2 volumes). After resuspension in 10 mM Tris-HCl-1 mM EDTA, pH 8, the DNA concentration was determined by absorption of UV light at 260 nm. DNA (20 µg) was electrophoresed (Fisher Biotech System, Fisher Scientific) at 70 V in a 1.8% agarose gel in Tris-phosphate-EDTA buffer stained with SYBR Green and photographed with UV illumination. A 100-bp DNA ladder molecular-weight standard (GIBCO BRL) was added to each gel as a reference for analysis of internucleosomal DNA fragmentation (43).

Electron microscopy. Confirmation of an LPS-induced apoptotic pathway and modification by overexpression of MnSOD was obtained by transmission electron microscopy. Endothelial cells were grown to confluence and treated with LPS (0.1 µg/ml, 4 h). Monolayers were washed with phosphate-buffered saline, fixed in 2.5% glutaraldehyde for 1 h, dehydrated, and embedded for electron microscopy with standard techniques. Alternating ultrathin (70 nm) and semithin sections (250 nm) were cut with a Reichert Ultracut S microtome and mounted on glass slides and 200-mesh copper grids, respectively. The semithin sections were counterstained with 1% toluidine blue and observed by light microscopy. The ultrathin sections were selected at the midnuclear plane of cells that tightly adhered to the plastic surface and also, away from the surface, at the midplane of the nuclei of cells that were lifted from the dish (~2 µm). Selected thin sections were counterstained with 4% uranyl acetate and 2% lead citrate and were observed with a J EOL 100 CXII electron microscope.

Experimental Protocols

Effect of overexpression of iNOS on LPS-induced apoptosis in DFG-iNOS SPAECs. DFG-iNOS SPAECs were plated in 25-cm² tissue culture flasks with or without L-NMMA (1 mM). At 24-h intervals up to 4 days, DFG-iNOS SPAECs were exposed to LPS (0.1 µg/ml × 4 h), Escherichia coli 0111:B4; Sigma) followed by phase-contrast microscopy, Hoechst staining, and internucleosomal DNA fragmentation. We also made these latter determinations in DFG-iNOS SPAECs (with or without L-NMMA) that were not exposed to LPS.
Prolonged Synthesis of iNOS-Derived NO Reduces the Sensitivity of SPAECs to LPS-Induced Apoptosis

As recently reported (38), infection of SPAECs with high-titer DFG-iNOS supernatant and selection in G-418 resulted in stable DFG-iNOS SPAECs that had 1) high levels of iNOS mRNA that migrated at 7.5 kb as expected for the polycistronic retroviral transcript, 2) iNOS protein with mobility similar to that of endogenous human iNOS protein in the cytosol from cytokine-treated human hepatocytes, and 3) continuous nitrite production (155.0 ± 10.7 nmol·mg protein⁻¹·24 h⁻¹) that was reduced by L-NMMA (1 mM) to levels detected in wild-type or DFG-LacZ SPAECs (~6 nmol·mg⁻¹·24 h⁻¹). A similar level of nitrite production was noted in SPAECs infected with Ad.CMV-iNOS at a multiplicity of infection of 20 (37). Stimulation of wild-type SPAECs with cytokine combinations effective in inducing iNOS expression in other cell types failed to yield detectable levels of iNOS mRNA (data not shown).

LPS caused internucleosomal DNA fragmentation in DFG-iNOS SPAECs at 24, 48, and 72 h after removal from L-NMMA (Fig. 1, lanes 4–6). At 96 h, however, DFG-iNOS SPAECs were resistant to LPS (Fig. 1, lanes 3 and 7). There was no spontaneous detectable internucleosomal DNA fragmentation in DFG-iNOS SPAECs at 96 h with (Fig. 1, lane 1) or without (data not shown) L-NMMA; L-NMMA, however, restored the sensitivity of DFG-iNOS SPAECs to LPS-induced apoptosis (Fig. 1, lane 2). Confirmation of these results was obtained by counting the number of cells with condensed, clumped, or segmented chromatin (via Hoechst stain and fluorescence microscopy). In Fig. 2, we note a time-dependent decrease in sensitivity to LPS that was significant at 96 h after removal from L-NMMA and was prevented by L-NMMA (data not shown). This time-dependent delayed (96-h) resistance to LPS was noted in eight of nine subcultures of DFG-iNOS SPAECs and was reminiscent of the recent report by Tzeng et al. (37) in either Ad.CMV-iNOS- or SNAP-treated SPAECs. It is noteworthy that acute exposure to either the NO donor SNAP or the potential peroxynitrite generator 3-morpholinosydnonimine (SIN-1) did not affect LPS sensitivity but rather required daily exposure to SNAP.

Overexpression of SOD and Sensitivity of SPAECs to LPS-Induced Apoptosis

Because partially reduced oxygen species may be an early signal in apoptosis, we infected SPAECs with Ad.CMV-MnSOD, -Cu,ZnSOD or -LacZ. At 48–72 h postinfection, total SOD activity increased by almost threefold after either Ad.CMV-MnSOD or -Cu,ZnSOD but was similar to wild-type levels after Ad.CMV-LacZ.
Wong et al. (42) recently demonstrated by immunocytochemistry that human MnSOD transgene expression colocalized to mitochondria, whereas Cu,ZnSOD expression was cytoplasmic. In Fig. 3, we show the acute effects of LPS (0.1 µg/ml, 4 h) on DNA laddering in the various cell types. LPS caused apparent internucleosomal DNA fragmentation in Ad.CMV-Cu,ZnSOD, Ad.CMV-LacZ, and wild-type SPAECs. There was considerably less apparent DNA fragmentation in Ad.CMV-MnSOD SPAECs.

Electron microscopy revealed a relatively normal nuclear and cytoplasmic morphology in wild-type SPAECs (Fig. 4A) and Ad.CMV-MnSOD SPAECs after LPS (data not shown). Of note, however, mitochondria of LPS-treated wild-type SPAECs that remained attached to the plastic dish showed evidence of internal vacuolation (Fig. 4C, inset), whereas normal mitochondrial morphology was apparent in Ad.CMV-MnSOD SPAECs after LPS (Fig. 4B). In addition, there was mitochondrial vacuolation present in wild-type SPAECs under control conditions (Fig. 4A, inset).

Effect of Overexpression of iNOS on Levels of MnSOD and Other Gene Products

Because a recent report (15) indicated that NO (and most likely peroxynitrite) may upregulate MnSOD, we determined the levels of expression of MnSOD in DFG-iNOS SPAECs. There was no apparent time-dependent change in mRNA of MnSOD nor was there an effect of L-NMMA at any time period from 24 to 96 h (Fig. 5A). Regulation of MnSOD expression is complex, and thus we examined levels of immunoreactive MnSOD.

**Fig. 2.** LPS-induced apoptosis is decreased in a time-dependent fashion after synthesis of NO in DFG-iNOS SPAECs. Apoptosis was quantified morphologically. A: DFG-iNOS SPAECs synthesized NO continuously (without L-NMMA; control), and apoptosis caused by LPS decreased as more time was allowed for NO biosynthesis after L-NMMA was withdrawn. B: LPS-induced apoptosis is sensitive to L-NMMA in DFG-iNOS SPAECs studied at 96 h. *P < 0.05 compared with control.

**Fig. 3.** Overexpression of Mn superoxide dismutase (MnSOD) but not Cu,ZnSOD inhibits LPS-induced internucleosomal DNA fragmentation. Standard 1.8% agarose gel electrophoresis shows internucleosomal DNA fragmentation at 4 h after LPS (0.1 µg/ml) in wild-type SPAECs (Ctrl + LPS) that is not apparent at control (Ctrl). Neither adenoviral gene transfer of ß-galactosidase (Ad.CMV-LacZ; LacZ) nor Cu,ZnSOD (Ad.CMV-Cu,ZnSOD; Cu,ZnSOD + LPS) affected LPS-induced DNA laddering. In contrast, overexpression of MnSOD after adenoviral gene transfer reduced sensitivity of SPAECs to LPS-induced DNA laddering (MnSOD + LPS). Adenoviral infection by itself did not result in detectable levels of DNA laddering (LacZ). Lanes at left and right, DNA 100-bp ladders.
by Western blot (Fig. 5B). MnSOD migrated to the appropriate molecular weight in the Western blot of Fig. 5. Although MnSOD protein increased with time, there was no effect of L-NMMA at any of the time points in this and two other blots. MnSOD activity has recently been shown to be decreased by products of NO and superoxide anion (23), and thus we also quantified MnSOD enzyme activity. MnSOD activity increased (Fig. 5C), but like the immunoreactive protein levels in Fig. 5B, there was no significant effect of L-NMMA at any time point, suggesting that induction of MnSOD expression did not underlie the delayed protection of iNOS-derived NO. Similar negative results regarding MnSOD protein levels were noted in Ad.CMV-iNOS SPAECs (data not shown).

We then surveyed several other candidate proteins that could potentially inhibit LPS-induced apoptosis and might also be induced by NO. In Fig. 6, we note that immunoreactive Bd-2 was detectable in similar levels in either control wild-type SPAECs or SNAP-treated SPAECs after 96 h. In addition, Bd-2 was detectable in similar levels in Ad.CMV-LacZ SPAECs or Ad.CMV-iNOS SPAECs without or with L-NMMA. Acute exposure (4 h, 10 µg/ml) to LPS did not affect Bd-2 levels in any of these respective conditions (Fig. 6). HSP70 levels were detectable in DFG-iNOS SPAECs but were not affected in a time-dependent or L-NMMA-sensitive fashion (Fig. 7). In addition, there was no detectable MT mRNA or HO-1 in DFG-iNOS SPAECs from 24 to 96 h with or without L-NMMA (data not shown). In this latter regard, tin protoporphyrin (an HO inhibitor) did not restore the sensitivity of DFG-iNOS SPAECs to LPS-induced DNA fragmentation at 96 h without L-NMMA (data not shown).

Because other cells appear capable of synthesizing a biologically active factor that is stable in the medium and inhibits pulmonary endothelial cell apoptosis (40), we tested whether such a factor was produced in the medium by the continuous synthesis of NO in DFG-iNOS SPAECs (Fig. 8). This was accomplished by collecting the supernatant (conditioned medium) from DFG-iNOS SPAECs 96 h after L-NMMA had been removed. Subsequently, wild-type SPAECs were exposed to the conditioned medium and treated with LPS (0.1 µg/ml × 4 h). LPS-induced internucleosomal DNA fragmentation was apparent in wild-type SPAECs or SPAECs grown in conditioned medium. Therefore, the prolonged synthesis of NO via overexpression of iNOS by DFG-iNOS SPAECs does not appear to produce a soluble factor that inhibits LPS-induced apoptosis.

Effect of iNOS-Derived NO on LPS-Induced Changes in the ICE-Like Activity

Last, we turned our attention toward CPP32, an ICE-like protease, that has been suggested as a convergence point in apoptosis in many cells including basic fibroblast growth factor withdrawal-induced apoptosis in endothelial cells (21). We first determined that iNOS-derived NO did not affect LPS signaling at an early step by quantifying the acute LPS-induced changes in MnSOD mRNA in DFG-iNOS SPAECs (with or without L-NMMA) at 24–96 h. In the Western blot shown in Fig. 9, we note that LPS readily induces immunoreactive MnSOD in DFG-iNOS SPAECs independent of the time of NO biosynthesis (24–96 h). Thus although the receptors and signaling mechanism that couple LPS to altered SPAEC gene expression are unclear, at least those that account for changes in MnSOD activity remain intact.

In Fig. 10, we report relative LPS-induced changes in CPP32-like activity before and after LPS 2 µm above plastic dish (D) revealed an apoptotic nuclear morphology (D, inset). There were no detectable nuclear profiles of LPS-treated Ad.CMV-MnSOD SPAECs in this plane consistent with prevention of LPS-induced apoptosis in these transfected cells. LPS caused mitochondrial vacuolation in wild-type cells only (C, inset, arrowheads), whereas mitochondria of Ad.CMV-MnSOD SPAECs appeared normal after LPS (B, inset). There was vacuolation of some mitochondria of wild-type SPAECs in control conditions (A, inset, arrowheads). Bar in A, 5 µm (same for B and C). Bar in D, 0.5 µm. Bars in insets, 0.5 µm.
CPP32-like activity such that by 96 h of NO biosynthesis, LPS increased CPP32-like activity to only 0.33 ± 0.03 Abs mg⁻¹·h⁻¹ (Fig. 10A). In one experiment in DFG-iNOS SPAECs (performed in triplicate) when NO biosynthesis occurred by withdrawal of L-NMMA, CPP32-like activity after LPS was 83, 82, 40, and 39% at 24, 48, 72, and 96 h, respectively, when normalized to CPP32-like changes in activity in the presence of L-NMMA. LPS resulted in similar declining increases in CPP32-like activity in SPAECs treated with SNAP on a daily basis. The effect of NO in decreasing LPS-induced activation of CPP32-like activity was apparent in the sensitivity of such inhibition to L-NMMA. At 96 h, LPS increased CPP32-like activity in Ad.CMV-iNOS SPAECs to only 37% of that noted after LPS exposure to wild-type SPAECs (Fig. 10A). Addition of 1 mM L-NMMA restored this response to 86% of the control value (Fig. 10B). Addition of DTT (20 mM) to lysates after LPS exposure did not significantly affect CPP32-like activity in wild-type or L-NMMA-treated Ad.CMV-iNOS SPAECs. DTT did, however, significantly increase CPP32-like activity in LPS-treated Ad.CMV-iNOS SPAECs at 96 h (without L-NMMA).

DISCUSSION

In the present study, we add to the growing body of information that exposure of cells to low doses of NO reduces their sensitivity to subsequent oxidative or nitrosative stress. We utilized a retroviral vector with an amphotropic backbone and a polycistronic intron (DFG) that facilitated expression of two genes. This enabled us to engineer stable transfectants of SPAECs in which all cells expressed human iNOS after selection in G-418. Overexpression of iNOS resulted in a time-dependent inhibition of LPS-induced apoptosis (Figs. 1 and 2). The 72- to 96-h period of continuous NO production required to affect LPS resistance in SPAECs suggested an NO-dependent induction of secondary protective molecules. Although direct adenoviral-mediated gene transfer of human MnSOD inhibited LPS-induced apoptosis in SPAECs (Figs. 3 and 4), we

Fig. 5. Time-dependent changes in MnSOD gene expression in DFG-iNOS SPAECs. A: Northern blot of MnSOD mRNA at 24–96 h of NO biosynthesis in DFG-iNOS SPAECs. There was no difference in hybridization signal of 2 detectable transcripts of MnSOD at any time point with or without NO biosynthesis. B: Western blot of MnSOD in protein isolated from DFG-iNOS SPAECs reveals a time-dependent increase in enhanced chemiluminescence that appears independent of NO biosynthesis (e.g., with or without L-NMMA). C: time-dependent increase in MnSOD enzymatic activity in DFG-iNOS SPAECs is not due to NO biosynthesis. Values are means ± SE. MnSOD activity in protein isolated from DFG-iNOS SPAECs reveals a time-dependent increase that is not sensitive to L-NMMA.

Fig. 6. Lack of effect of LPS and NO exposure of SPAECs on steady-state levels of immunoreactive Bcl-2. Bcl-2 was detected by Western blot under basal conditions (−) and 4 h after LPS (+) in wild-type SPAECs (lanes 1 and 2), SPAECs exposed to S-nitroso-N-acetylpenicillamine (SNAP) daily for 96 h (lanes 3 and 4), Ad.CMV-LacZ SPAECs (LacZ; lanes 5 and 6), or Ad.CMV-iNOS SPAECs without (iNOS; lanes 7 and 8) or with (iNOS + L-NMMA; lanes 9 and 10) L-NMMA.

Fig. 7. Immunoreactive 70-kDa heat shock protein (HSP70) is not induced in DFG-iNOS SPAECs after 24–96 h of NO biosynthesis. Western blot of HSP70 reveals a single band of enhanced chemiluminescence signal that remains constant after 24–96 h of NO biosynthesis in DFG-iNOS SPAECs and is not affected by L-NMMA at any time point studied. +, With L-NMMA; −, without L-NMMA.
were unable to detect L-NMMA-sensitive iNOS-derived NO induction of MnSOD mRNA (Fig. 5A), immunoreactive protein (Fig. 5B), or enzyme activity (Fig. 5C). Other candidate genes noted previously to inhibit LPS-mediated apoptosis [HSP70 (43); Fig. 7] or oxidant-mediated injury to endothelial [MT (31); HO-1 (30)] or other cells [Bcl-2 (12); Fig. 6] were likewise unaffected by NO biosynthesis. We did note, however, that NO biosynthesis was associated with inhibition of the LPS-mediated increase in the activity of caspase-3-like enzyme (Fig. 10A) in a DTT-sensitive fashion (Fig. 10B), suggesting an important role for S-nitrosylation of this particular cysteine protease in the apoptotic signaling pathway in pulmonary endothelium.

Overexpression of iNOS Inhibits LPS-Mediated Apoptosis

Like wild-type SPAECs (14), SPAECs infected with human iNOS in the presence of L-NMMA (Fig. 1) undergo internucleosomal DNA fragmentation within 4 h of exposure to LPS (0.1 µg/ml). We also used changes in nuclear morphology (Fig. 2) and electron microscopy (Fig. 4) to demonstrate LPS-induced apoptosis in SPAECs. Other manifestations of apoptosis, including large-molecular-weight DNA fragmentation and DNA strand breaks were noted previously (13, 14). Infection of cells with DFG-iNOS (Figs. 1 and 2), adenoviral-mediated transfer of iNOS or NO donors including SNAP (35), or SIN-1 (data not shown) did not, in themselves, induce apoptosis. In this regard, it appears that SPAECs are resistant to NO toxicity because Razzack et al. (34) and Tzeng et al. (37) previously demonstrated that NO biosynthesis after gene transfer did not affect endothelial cell chromium release, thymidine uptake, or proliferation.

Although we did not utilize clonal expansion of retroviral-infected SPAECs, we did note similar levels of nitrite synthesis from several different infections as well as from several subpassages of infected cells (>10 determinations on different occasions). Indirect comparison of NO biosynthesis after DFG versus adenoviral infection with nitrite accumulation revealed that each approach resulted in ∼100 nmol nitrite · mg protein⁻¹ · 24 h⁻¹ (37). This similarity occurred despite the fact that only 20–30% of the adenoviral-infected cells expressed transgene, whereas all the DFG-iNOS SPAECs were positive for immunoreactive iNOS (38). This suggests that similar phenotypes in DFG- or Ad.CMV-iNOS-infected cells (e.g., time-dependent decreased sensitivity to LPS) were the result of diffusion of NO or its active chemical congener.

The time-dependent delay in resistance to LPS-induced apoptosis is clearly due to NO biosynthesis. It is L-NMMA sensitive (Figs. 1 and 2) and did not occur after an irrelevant vector such as Ad.CMV-LacZ rather than Ad.CMV-iNOS was used (35). Similar NO biosynthesis after either retroviral or adenoviral vector transfer of human iNOS to SPAECs resulted in similar degrees of delayed protection. Thus two highly dissimilar vectors (DFG and Ad.CMV) expressing the same transgene (e.g., human iNOS) resulted in the same phenotype in SPAECs. In the previous report by Tzeng et al. (37), the time-dependent effect could be mimicked by daily exposure to modest amounts of the chemical donor SNAP. Thus all evidence to date strongly supports a role for continuous production of low amounts of NO as cytoprotective (39). NO has been proposed to be cytoprotective against oxidant injury by virtue of its ability to terminate the propagation of radical-medi-
ated lipid peroxidation (35), scavenge superoxide anions (8), or reduce toxic ferryl species to ferrous iron, thereby blocking hemoprotein-mediated Fenton-like reactions (19). All of these mechanisms involve direct chemical interactions of NO with target molecules, and thus it is difficult to account for the prolonged delay in protection. Alternatively, NO is known to induce a number of genes (10), and several of these are candidates as inhibitors of LPS-induced apoptosis. Accordingly, we focused on revealing the roles of several candidate NO-sensitive genes as antiapoptotic molecules and then sought a link to the present study by determining their inducibility by NO.

**Overexpression of MnSOD Inhibits LPS-Induced Apoptosis But Synthesis of NO After Gene Transfer Does not Affect MnSOD Levels**

We initially focused on a role for MnSOD as the NO-dependent gene product that reduces LPS-induced apoptosis in SPAECs. MnSOD is known to be an oxidant-sensitive gene that increases in response to partially reduced oxygen (29) and nitrogen (15) species in cultured lung cells. Furthermore, overexpression of MnSOD reduces the sensitivity of cells to TNF-α (42), a potentially important signaling molecule in the LPS response; it has been suggested that overexpression of MnSOD may likewise inhibit LPS-mediated endothelial cell toxicity (27).

We noted that overexpression of MnSOD, but not of Cu,ZnSOD, inhibited LPS-induced apoptosis in SPAECs (Fig. 3). Because the levels of overall SOD activity increased in a similar fashion after adenoviral gene transfer of either human Mn- or Cu,ZnSOD, it is quite likely that the mitochondrial compartmentalization of the former transgene (46) accounts for its specific antiapoptotic effects. Considerable attention has been focused on this particular subcellular compartment in the process of apoptosis as it relates to the roles of cytochrome c and antioxidants such as Bcl-2 (12, 20, 44). The contribution of oxidative stress to apoptotic signaling and the potential importance of mitochondrial events in the early steps of apoptosis clearly underscore the potential role of MnSOD. In this regard, it was noteworthy that overexpression of MnSOD not only protected SPAECs against LPS-induced apoptosis (Figs. 3 and 4) but resulted in nonapoptotic SPAECs that remained attached to the plastic culture plate having more robust, morphologically normal mitochondria (Fig. 4B, inset) than even wild-type SPAECs.

Although the nuclear morphology of wild-type SPAECs that were exposed to LPS (Fig. 4C) and remained attached to the plastic dish were relatively normal at an electron-microscopic level, their mitochondria appeared to have substantial cisternal vacuolation (see Fig. 4C, inset). This suggests that a portion of the LPS-treated SPAECs that were attached to their substratum may have been in a preapoptotic stage as manifested by altered mitochondria. Accordingly, this is the first direct demonstration that overexpression of MnSOD protects lung cells against LPS-mediated apoptosis and that early changes in endothelial cell proapoptotic pathways may be apparent in the structural changes in their mitochondria. The role of MnSOD in protecting lung cells against other forms of oxidant-mediated injury is less clear. For example, overexpression of MnSOD with a β-actin promoter in transgenic mice resulted in a very modest resistance to 90% oxygen (11) in contrast to resistance to 99% oxygen in mice overexpressing MnSOD with a surfactant protein C promoter (41).
creased by NO biosynthesis. Unlike a preliminary report (15) indicating that peroxynitrite can induce MnSOD at a transcriptional level in cultured lung epithelial cells, when NO was synthesized after DFG-mediated gene transfer, there was no l-NMMA-sensitive change in MnSOD mRNA (Fig. 5A), immunoreactive protein (Fig. 5B), or enzyme activity (Fig. 5C) at any time point studied. The approximate twofold increase in MnSOD protein and activity noted over time was not sensitive to l-NMMA, suggesting that MnSOD expression was not affected by continuous production of low amounts of NO. Thus genotypic or phenotypic changes noted after NO exposure may be specific with respect to the kinetics of NO release. Large amounts of NO derived from a chemical donor over short periods of time may not be predictive for changes noted after lower amounts of NO are synthesized at a more constant rate. Alternatively, the balance between NO and superoxide anions may have precluded production of sufficient amounts of peroxynitrite to affect MnSOD expression (15) or MnSOD activity (23).

Effect of NO Biosynthesis on Cytoprotective Proteins in Cultured Lung Endothelial Cells

Because induction of MnSOD did not appear to be the mechanism by which NO was cytoprotective in SPAECs, we determined the effect of prolonged NO biosynthesis on other candidate genes that were either proapoptotic, antioxidant, or potentially induced by NO (or all three). We initially focused on HSP70 because our group has shown that three). We initially focused on HSP70 because our group has shown that direct gene transfer of HSP70 (Fig. 6). Bcl-2 is a member of a large family of homologous proteins including BAX (a proapoptotic molecule), and a lack of change in steady-state Bcl-2 immunoreactive protein may not be predictive of cell fate because the ratio of these cofactors may contribute to their roles in physiological contexts.

We also sought a role for the oxidant-sensitive (5) stress gene MT because our group recently demonstrated that overexpression of MT after direct gene transfer reduces the sensitivity of SPAECs to oxidative injury (31) including LPS-mediated apoptosis (34). There was no detectable level of MT mRNA by Northern blot analysis after NO biosynthesis (data not shown). We did not measure MT protein. Although it is possible that we missed a transient increase in MT mRNA, in general, MT mRNA levels are readily detectable before significant increases in MT protein, including a recent experience by Pitt et al. (31) with cadmium exposure in SPAECs.

We also performed a bioassay to determine whether there were any unknown stable products secreted into the medium by DFG-iNOS-infected SPAECs that could be transferred to wild-type SPAECs, resulting in a decrease in their sensitivity to LPS. Such an unknown factor was suggested to be secreted by alveolar type II cells, rendering cultured pulmonary artery endothelial cells resistant to TNF-α-induced apoptosis (40). Transfer of medium from DFG-iNOS SPAECs conditioned for 24–96 h, however, did not affect LPS-induced apoptosis in wild-type SPAECs (Fig. 8). These data suggest that the protective factor(s) is intracellular or unstable in cell culture medium.

Inhibition of LPS-Induced Apoptosis in SPAECs Is Associated With Decreased Activation of Caspase-3-Like Protease Activity

We performed additional experiments to assess the signaling step that may be affected by NO biosynthesis in LPS-induced apoptosis. Because LPS is known to bind to a receptor on endothelial cells, generate reactive oxygen species, and increase transcription of MnSOD (29), we determined whether these components of LPS-induced apoptosis were intact after 24–96 h of NO biosynthesis in DFG-iNOS SPAECs. In Fig. 9, it is apparent that MnSOD mRNA increased in response to LPS at all time intervals with or without l-NMMA. Thus we assume that the inhibition noted at 96 h regarding apoptosis (Figs. 1 and 2) was not secondary to loss of LPS-receptor function or the signaling elements involved in induction of MnSOD (e.g., TNF-α or reactive oxygen intermediates). Because activation of ICE-like cysteine proteases was linked to endothelial cell apoptosis after growth factor withdrawal (21) and Tzeng et al. (37) recently reported that LPS-induced apoptosis in SPAECs is sensitive to Ac-DEVD-CHO, a caspase-3-specific inhibitor, we determined changes in caspase-3-like activity after LPS in Ad.CMV-iNOS and DFG-iNOS SPAECs. We hypothesized that NO inhibition of LPS-induced apoptosis would be associated with a lack of activation of caspase-3-like enzyme activity as suggested by preliminary data by Tzeng et al. (37) in SPAECs as well as recent reports suggesting that 1) endothelial NOS-derived NO interfered with...
TNF-α-induced apoptosis in endothelial cells through mechanisms involving suppression of caspase-3-like protease activity (6) and 2) iNOS-derived NO interfered with apoptosis in cultured rat and mouse hepatocytes, in part, via S-nitrosylation of CPP32-like proteases (19). In the present study, LPS increased CPP32-like activity in wild-type SPAECs, and this LPS-induced increase declined during the 96-h interval in which Ad.CMV-iNOS- or DFG-iNOS SPAECs synthesized NO (Fig. 10A). This inhibition of LPS-induced activation of CPP32-like activity was sensitive to L-NMMA (Fig. 10B) and was reversible in vitro with the addition of DTT to the lysate (Fig. 10B). Thus prolonged synthesis of NO results in S-nitrosylation of caspase-3-like enzyme activity that apparently is associated with decreased apoptosis. Although Kim et al. (19) and others (26) recently reported that NO can directly inhibit caspase-3-like activity through protein S-nitrosylation, the lack of acute (~24-h) effects of NO donors (including SNAP or SIN-1) on LPS-induced apoptosis and the prolonged period of time (72–96 h) required to develop resistance to LPS suggests that the effects of NO on CPP32-like activity are complex and involve direct protein S-nitrosylation and more indirect effects that may require more prolonged periods of exposure to NO.

In summary, continuous synthesis for 96 h of relatively low levels of NO after either Ad.CMV- or DFG-iNOS infection of SPAECs inhibits LPS-induced apoptosis. The time-dependent nature of this phenomenon suggests that NO may be inducing expression or activity of other cellular genes, although neither MnSOD, HSP70, MT, HO-1, nor Bd-2 can account for our results. Target sites for such suppression do not appear to include the putative LPS receptor or signaling molecules thought to account for LPS-induction of MnSOD (e.g., reactive oxygen intermediates or cytokines such as TNF-α). An association of low-level NO synthesis and S-nitrosylation of a pivotal molecule (caspase-3-like cysteine protease) in initial signaling in LPS-induced apoptosis is apparent. Thus the relative rates of NO synthesis (in conjunction with concurrent levels of superoxide anion and/or intracellular thiols) and the role of caspase-3-like enzymes may determine the balance in anti- vs. proapoptotic signaling.

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REFERENCES


