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Apical, But Not Basolateral, Endotoxin Preincubation Protects Alveolar Epithelial Cells Against Hydrogen Peroxide-Induced Loss of Barrier Function: The Role of Nitric Oxide Synthesis

Frank Rose,* Bernd Guthmann,* Tobias Tenenbaum,* Ludger Fink,* Ardeschir Ghofrani,* Norbert Weissmann,* Peter König,† Leander Ermert,* Gabriele Dahlem,* Joerg Haenze,* Wolfgang Kummer,† Werner Seeger,* and Friedrich Grimminger†*

The influence of LPS preincubation on hydrogen peroxide (H$_2$O$_2$)-induced loss of epithelial barrier function was investigated in rat alveolar epithelial type II cells (ATII). Both apical and basolateral H$_2$O$_2$ administration caused a manifold increase in transepithelial $[^{3}H]$mannitol passage. Apical but not basolateral preincubation of ATII with LPS did not influence control barrier properties but fully abrogated the H$_2$O$_2$-induced leakage response. The effect of apical LPS was CD14 dependent and was accompanied by a strong up-regulation of NO synthase II mRNA and protein and NO release. Inhibition of NO by N$\textsuperscript{G}$-monomethyl-l-arginine suppressed the LPS effect, whereas it was reproduced by exogenous application of gaseous NO or NO donor agents. Manipulation of the glutathione homeostasis (buthionine-$\textsuperscript{S,R}$- sulfoximine) and the cGMP pathway (1H-(1,2,4)oxadiazolo[4,3-$\alpha$]quinoxaline-1-one; zaprinast) did not interfere with the protective effect of LPS. Superoxide (O$_2^-$) generation by ATII cells was reduced by exogenous NO and LPS preincubation. O$_2^-$ scavenging with exogenous superoxide dismutase, the intracellular superoxide dismutase analog Mn(III)tetakis(4-benzoic acid) porphyrin, and the superoxide scavenger nitroblue tetrazolium and, in particular, hydroxyl radical scavenging with hydroxyl radical scavenger 1,3-dimethyl-thiourea inhibited the H$_2$O$_2$-induced epithelial leakage response. In conclusion, apical but not basolateral LPS preincubation of ATII cells provides strong protection against H$_2$O$_2$-induced transepithelial leakage, attributable to an up-regulation of epithelial NO synthesis. It is suggested that the LPS-induced NO formation is effective via interaction with reactive oxygen species, including superoxide and hydroxyl radicals. The polarized epithelial response to LPS may be part of the lung innate immune system, activated by inhaled endotoxin or under conditions of pneumonia. The Journal of Immunology, 2002, 169: 1474–1481.

Activation of polymorphonuclear leukocytes has long been implicated in the development of acute respiratory distress syndrome (ARDS),$^3$ characterized by gas exchange abnormalities and pulmonary edema formation due to increased lung endothelial and epithelial permeability (1–4). Hydrogen peroxide (H$_2$O$_2$), a reactive oxygen species, is an established activator of polymorphonuclear leukocyte-derived mediator of epithelial injury. In alveolar epithelial cells, H$_2$O$_2$ effects are manifold and include an initial depletion of intracellular ATP, a sustained inhibition of alveolar epithelial type II cell (ATII) surfactant synthesis, loss of epithelial barrier properties, DNA damage, and cell lysis at high dosage (5–9).

Sepsis is the leading cause of mortality in critically ill patients (10) and represents an important underlying event in the pathogenesis of ARDS. In animal models and under cell culture conditions, various clinical features of ARDS may be reproduced by endotoxin (LPS) released from cell walls of Gram-negative bacteria (1–3). The microcirculatory disturbances with subsequent cellular injury and loss of organ function induced by LPS in intact animals have been attributed to the activation of inflammatory cells and strong inflammatory mediator generation. ATII exposed to LPS demonstrated a marked depression of surfactant synthesis and ion transport as well as strong up-regulation of adhesion molecules, cytokines such as TNF-$\alpha$, and NO synthesis via NO synthase II (NOSII) (11–14).

At the interface between external environments and the milieu interior, the alveolar lining layer has developed a highly organized cellular polarity: surfactant components, as an example, are exclusively secreted to the apical side, whereas fibrinogen is mainly secreted basolaterally (15). Moreover, in response to inflammatory challenge, the alveolar epithelium orchestrates enhanced leukocyte traffic to the apical side by polarized chemokine secretion and up-regulation of adhesion molecules (16), whereas protein transport across rat alveolar epithelial cell monolayers displays asymmetry with predominance of the apical-to-basolateral flux (17), the same being true for the active transepithelial sodium transport (18, 19).

In the present study in ATII monolayers, we investigated the impact of apically vs basolaterally applied endotoxin on the H$_2$O$_2$-elicited epithelial leakage response. Interestingly, a protective role of LPS pretreatment was noted; however, it was restricted to the...
apical route of LPS administration. Up-regulation of epithelial NOSII-driven NO synthesis was identified as a predominant mechanism underlying the protective effect of endotoxin preincubation, and evidence is presented that NO exerts its function largely via interaction with oxygen radicals, with superoxide anion and hydroxyl radicals putatively appearing as downstream effectors of H₂O₂.

Materials and Methods
Male CD18 Sprague-Dawley rats (180–200 g) were purchased from Charles River (Sulzfeld/Main, Germany). Elastase (type EC 134, sp. act. 135 U/mg protein) was obtained from Elastin Products (St. Louis, MO). DMEM was supplied by Life Technologies (Karlsruhe, Germany). Zaprinast, Zn(II)-protoporphyrin, manganese-(III)-tetraakis(benzoic acid) porphyrin chloride, desferrioxamine, 1,3-dimethylthiourea, reduced glutathione (GSH), buthionine-(S,R)-sulfoximine (BSO), hydrogen peroxide (urea stabilized), HRP, 8-bromoadenosine (8-Br)-cGMP, LPSs from Salmonella typhimurium and IgG2b Ab were obtained from Sigma (Deisenhofen, Germany). 5-Nitroso-N-acetyl-t-phenylalanine (SNAP) and spermere NONOate were purchased from Calbiochem (La Jolla, CA). [1H]Methylcholine, 511-labeled cGMP assay system was from Amersham (Dreieich, Germany). The Limulus assay system was observed by Hemochrom (Essen, Germany). The CD14 Ab (MY-4, monoclonal) was from Coulter (Krefeld, Germany). Tissue culture plastic was purchased from BD Biosciences (Heidelberg, Germany).

Isolation of ATII
ATII were isolated as previously described in detail (20). Briefly, inflated and perfused lungs from specific pathogen-free male CD18 Sprague-Dawley rats were lavaged and filled to their total lung capacity with elastase (1 mg/ml) and trypsin (0.05 mg/ml)-containing solution. Lungs were minced and free cells were separated from lung tissue by sequential filtration through 100-μm and additional 10-μm sterile nylon mesh. "Panning" of the resultant cell suspension was performed on rat IgG-coated plates. Non-adherent ATII were harvested after 1 h and resuspended in DMEM containing 10% FCS. The yield of type II epithelial cells from each rat was in the range of 30–50 × 10⁶. The percentage of freshly isolated type II cells was 94 ± 2% as assessed by modified Papanicolaou, tannic acid, and alkaline phosphatase staining. Contaminated cells included alveolar macrophages (<4%), and neutrophils (<2%). After culture of the cells for 3 days on transwells, the electric resistance of all ATII layers used was >2000 Ω/cm². In this state the percentage of type II cells was virtually 100%, as assessed by electron microscopy in separate control experiments. Using MACS in additional experiments, highly purified type II cell cultures were manufactured and used to detect the NOSII protein in type II cells by immunofluorescence (anti-rat NOSII; Santa Cruz Biotechnology, Santa Cruz, CA) and the liberation of NO. MACS was performed with anti-rat CD45 (leukocyte common Ag) Ab (OX-1; BD PharMingen, San Diego, CA) and confirmed by immunofluorescence with anti-rat macrophage Ab (ED-1; DPC Biermann, Bad Nauheim, Germany) and anti-rat CD45 Ab. ATII viability, as assessed by 5-carboxyfluorescein diacetate loading and trypan blue exclusion, was persistently >95%. Experiments have been performed in the presence of 1% FCS.

Determination of paracellular permeability
For measuring epithelial permeability, [1H]mannitol (1 μCi/ml) was added to the apical side during the incubation period.

mRNA extraction
Aliquots of 1 × 10⁶ cells were transferred into 1.5-ml reaction tubes. After centrifugation at 300 × g, the supernatant was removed and the pellet was lysed in 300 μl lysis buffer of the Dynabeads mRNA direct kit (Dynal Biotech, Oslo, Norway). For each sample, 150 μg beads were applied. Isolated mRNA was finally solved in 20 μl diethyl pyrocarbonate-treated H₂O.

Relative mRNA quantification
Relative mRNA quantitation was performed by the Sequence Detection System 7700 (PE Applied Biosystems, Foster City, CA) and real-time PCR. We used comparative quantitation (ΔCt) normalizing target gene to an internal standard gene, as described in detail by Fink et al. (21). For internal calibration, mRNA transcribed from the gene encoding porphobilinogen deaminase (PBGD) was used. We could show that amplification efficiency of PBGD and NOSII primer/probe sets was approximately equal and amounted to 0.95 ± 0.02 (95 ± 2%).

cDNA synthesis and real-time PCR
For cDNA synthesis and real-time PCR, reagents as well as primers and probes were applied as described by Fink et al. (22). Two microliters of cDNA were applied to each sample. Primers were added to a final concentration of 300 nM each and hybridization probes were added to a final concentration of 200 nM in a volume of 50 μl. Cycling conditions were 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 61°C for 60 s.

Measurement of cGMP
ATII were cultured on 35-mm dishes at a density of 3 × 10⁶ cells per well (plating density was typically 4 × 10⁵/cm²). Measurement of cyclic nucleotides was performed with a commercially available radioligand assay in the supernatant of permeated cells, subjected to 70% ethanol for 60 min.

Measurement of O₂⁻ concentration
Superoxide production in ATII was measured by lucigenin-induced chemiluminescence as recently described by Höhler et al. (23).

Measurement of H₂O₂
H₂O₂ was measured as described previously by Dekhuijzen et al. (24). Briefly, 100 μl of 420 μM 3',5',5'-tetramethylbenzidine (dissolved in 0.42 M citrate buffer, pH 3.8) and 10 μl of 52.5 U/ml HRP solution were added to 100 μl of the probe. The reaction was terminated after 20 min by admixture of 10 μl 18 N sulfuric acid. Solutions were measured photometrically by 450 nm.

Measurement of cellular NO release
Cells were cultured in transwell dishes in DMEM for 24 h. For measurement of cellular NO release the gaseous headspace was forwarded to a chemiluminescence NO analyzer (Sievers 280 NOA; Sievers Instruments, Boulder, CO) for quantification of its NO concentration. Due to the very low liquid-gas partition coefficient of NO, NO released into the culture medium is expected to largely escape into the gaseous headspace (25).

Measurement of lactate dehydrogenase
Lactate dehydrogenase release as an indicator of cellular damage was quantified by standard colorimetric technique. It ranged ~2% of total enzyme activity in control cells as compared with the total release in response to the pore-forming agent mellitin (100 μg/ml).

Control experiments
The passage of LPS through the semipermeable culture membrane was measured by the Limulus assay system. Independent of the route of application, ~90% of the apically or basolaterally applied LPS was found to cross the transwell within 3 h.

Statistical analysis
For statistical comparison, one-way analysis of variance was performed. The Student Newman-Keuls test was used as a posteriori tests for linear contrasts. The significance level for the test was set at p = 0.05. A level of p < 0.05 was considered significant.

Results
Apical but not basolateral LPS suppress the epithelial leakage response to H₂O₂
Exposure of ATII to H₂O₂ induced a dramatic increase in epithelial permeability, as assessed by [1H]mannitol transit (Fig. 1). A maximum effect was observed upon basolateral Η₂Ο₂ (100 μM) application, whereas the apical route of application was less effective. Control cells did not change their epithelial permeability under influence of LPS. Apical preincubation of ATII with LPS for 12 h suppressed the toxic effect of both apically and basolaterally applied Η₂Ο₂ in a dose-dependent fashion (optimum concentration, 10 ng/ml LPS) (Fig. 2). This effect commenced after 6 h of LPS incubation, reaching a maximum within 12 h (data not given in detail). In contrast, basal pretreatment with LPS had no effect on H₂O₂-induced oxidative stress over the 12-h observation period.
Apical but not basolateral LPS application induces NOSII mRNA expression

In accordance with the protective effect on paracellular permeability, only apical incubation of ATII with LPS increased NOSII mRNA. This response was clearly dose dependent, with a maximum at 10 ng/ml LPS (23.55 ± 9% k × NOSII copies per copy of PBGD). Quantitative analysis via real-time PCR revealed an increase in NOSII mRNA up to a 145-fold level when compared with basal LPS application (1 ng/ml) (Fig. 3). mRNA expression in response to apical LPS incubation was markedly reduced in the presence of CD14 Abs, indicating that the LPS-induced NOSII expression proceeds via CD14-related signaling (Fig. 3). Correspondingly, LPS significantly increased the liberation of NO, assessed by chemiluminescence (Table I), and induced the expression of NOSII protein, as detected with immunofluorescence (Fig. 4). In contrast, in the absence of LPS, no NO release and NOSII protein expression was noted.

The role of NO in $H_2O_2$-induced increase in paracellular permeability

Pretreatment with the competitive NO synthase inhibitor $N^\omega$-monomethyl-L-arginine (L-NMMA; 1 mM) significantly reduced the protective effect of LPS preincubation, whereas both incubation of ATII in a NO atmosphere (250 ppm) or pretreatment with the NO donor agents spermine NONOate and SNAP reproduced the protective effect of LPS (Fig. 5A). The effect was reproducible by coincubation with both NO donors, whereas posttreatment did not ameliorate $H_2O_2$-induced hyperpermeability (data not given in detail). Interestingly, in the concentration range used, gaseous NO...
in the absence of H₂O₂ exerted some permeability-enhancing effect by itself (Fig. 5B).

To control the NO effect in an unrelated system, increased paracellular epithelial permeability was provoked by either *Escherichia coli* hemolysin, a membrane-perturbing bacterial exotoxin, or the polycation protamine. In contrast to H₂O₂, the epithelial leakage response to these two agents was not significantly influenced by NO or the NO donors NONOate and SNAP (data not given).

**How does LPS-induced NO protect against the H₂O₂-induced permeability increase?**

**Independence of cGMP.** Pretreatment of ATII with both the specific phosphodiesterase (PDE) type V inhibitor zaprinast (10 μM) as well as with the guanylate cyclase inhibitor 1H-(1,2,4)oxadiazolo[4,3-α]quinoxaline-1-one (ODQ, 10 μM) for influencing the epithelial cGMP levels did not affect the protective effect of LPS preincubation (10 ng/ml for 12 h, Fig. 6). In addition, application of 8-Br-cGMP over a range from 10 μM to 10 mM did not interfere with the toxic effect of H₂O₂, indicating that the protective mechanism of LPS/NO was independent of the cGMP-related signal transduction pathway. In preceding pilot experiments, the significant modulation of the intracellular cGMP level in the presence of zaprinast (increase in ATII ascertained) was shown.

**Independence of glutathione synthase and hemoxygenase activities.** Pretreatment of ATII with the glutathione synthase inhibitor BSO or the hemoxygenase inhibitor Zn-(II)-protoporphyrin did not reduce the LPS-induced protection against the H₂O₂-induced epithelial leakage (Fig. 7).

**Putative role of the superoxide anion (O₂⁻) and the hydroxyl radical (·OH).** The H₂O₂-induced increase in paracellular [³H]mannitol flux was significantly enhanced in the presence of optimum concentrations of the superoxide dismutase (SOD) inhibitor triethylenetetramine (TETA) (Table II). In contrast, SOD, the intracellular SOD analog Mn(III)tetrakis(4-benzoic acid) porphyrin (MnTBAP), and the O₂⁻ scavenger nitroblue tetrazolium (NBT) all reduced the H₂O₂-induced leakage, thus favoring a role of O₂⁻ in this event. Most prominent suppression of the H₂O₂-induced mannitol flux was achieved by the hydroxyl radical scavenger 1,3-dimethyl-thiourea (DMTU) (Table II).

Interestingly, cultured epithelial cells liberated substantial O₂⁻ release, as assessed by lucigenin chemiluminescence technique. This O₂⁻ liberation was suppressed by LPS treatment of the ATII cells and in the presence of the NO donor NONOate as well as the intracellular SOD analog MnTBAP (Table III).

**Putative role of H₂O₂ dissociation.** Testing the effect of NO on Fenton reactions, we measured H₂O₂ by a HRP assay in the presence and absence of spermine NONOate (10 μM) in vitro: coinubcation of Fe²⁺ (10 μM) and H₂O₂ (10 μM) decreased the amount of H₂O₂ to a level of ~35 ± 5% within 20 min as compared with controls in the absence of Fe²⁺. Application of spermine NONOate did not affect the degradation of H₂O₂ in the presence of Fe²⁺, indicating that this process was not directly influenced by NO.

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**Table I. Impact of LPS pretreatment on NO release in epithelial cells and macrophages**

<table>
<thead>
<tr>
<th>cells</th>
<th>NO Cellular Release (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Medium</td>
</tr>
<tr>
<td>ATII</td>
<td>0</td>
</tr>
<tr>
<td>ATII + 10% AΦ</td>
<td>0</td>
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</table>

*Type II cells (2 × 10⁶) were cultured in DMEM for 24 h in the presence and absence of LPS (10 ng/ml). Additionally, the same total number of ATII cells was enriched with 10% rat alveolar macrophages (AΦ). For measurement of cellular NO release, the gaseous headspace was forwarded to a Sievers 280 NOA chemiluminescence NO analyzer. Means ± SEM of three independent experiments each are given.

*p < 0.05.*
When investigating the impact of endotoxin preincubation on the H₂O₂-elicited epithelial leakage in ATII monolayers, an impressive but polarized response was noted. Whereas basolaterally applied LPS was entirely ineffective, apical LPS pretreatment provided strong protection, and this was largely attributable to enhanced epithelial NO synthesis. A CD14-dependent marked upregulation of NOSII was found in the ATII cells and the LPS effect was blocked by L-NMMA and was fully reproduced by exogenous administration of different NO donor agents as well as gaseous NO. Interestingly, the protective effect of NO apparently did not engage the epithelial guanylate cyclase pathway, as various approaches to manipulate the cGMP levels were ineffective. In contrast, evidence was forwarded for the hypothesis that NO exerted its function largely via interaction with oxygen radicals, with a putative role of hydroxyl radicals appearing as downstream effectors of H₂O₂.

**Discussion**

When investigating the impact of endotoxin preincubation on the H₂O₂-elicited epithelial leakage in ATII monolayers, an impressive but polarized response was noted. Whereas basolaterally applied LPS was entirely ineffective, apical LPS pretreatment provided strong protection, and this was largely attributable to enhanced epithelial NO synthesis. A CD14-dependent marked upregulation of NOSII was found in the ATII cells and the LPS effect was blocked by L-NMMA and was fully reproducible by exogenous administration of different NO donor agents as well as gaseous NO. Interestingly, the protective effect of NO apparently did not engage the epithelial guanylate cyclase pathway, as various approaches to manipulate the cGMP levels were ineffective. In contrast, evidence was forwarded for the hypothesis that NO exerted its function largely via interaction with oxygen radicals, with a putative role of hydroxyl radicals appearing as downstream effectors of H₂O₂.

During inflammatory diseases and ischemia-reperfusion, reactive O₂ species such as O₂⁻ and H₂O₂ may be produced in large quantities, exceeding endogenous antioxidant defenses and promoting tissue injury. Previous work to define the role of NO under these circumstances has produced variable results, which is particularly true for the lung. NO-related mechanisms that may protect against inflammatory injury in this organ include vasodilation (26–28) and suppression of leukocyte adhesion to the pulmonary endothelium as well as leukocyte activation (29). In lung ischemia-reperfusion models, inhalation of NO was shown to suppress the vasoconstrictor response and pulmonary edema formation, as well as ventilation-perfusion mismatch and shunt flow (30–34). In contrast, the rapid reaction of NO with O₂⁻ to form peroxynitrite may cause oxidation of protein and non-protein sulfhydryl groups and may enhance lipid peroxidation, thereby promoting tissue injury (35). Concerning the currently investigated alveolar epithelial
cells, NO/peroxynitrite was found to suppress the activity of sodium permeant cation and $\alpha$-type calcium channels on the apical surface, as well as the basolaterally located Na $\pm K^+$ ATPase (36, 37). Moreover, high concentrations of NO deteriorate the biophysical function of the type II cell-derived pulmonary surfactant system, which again was ascribed to NO-related peroxynitrite formation (38, 39), and NO was found to decrease ATP content and surfactant synthesis in freshly isolated ATII (40, 41).

Antioxidant defense systems fall into enzymatic and nonenzymatic categories. Previous examinations in ATII cells revealed the presence of SODs, catalase, and glutathione peroxidases (8, 42). The GSH-dependent pathway is, indeed, regarded as the major detoxification mechanism in the lung, because the GSH concentration in the epithelial lining layer is $\sim 100$ times higher than that commonly found in the extracellular fluid of various tissues, and $\gamma$-glutamyl transpeptidase is increased by oxidative stress in alveolar epithelial cells (42–45). It is thus tempting to speculate that the protective effect of LPS on the H$_2$O$_2$-elicited epithelial leakage response might be caused by an up-regulation of the ATII GSH-dependent antioxidant system; however, such a suggestion was not supported by the present experimental data. Pretreatment of the ATII cells with the glutathione synthase inhibitor BSO to deplete the epithelial glutathione pool did not interfere with the protective effect of LPS preincubation on the H$_2$O$_2$-induced epithelial leakage response.

In contrast, strong evidence was provided for the suggestion that an up-regulation of epithelial NO synthesis is primarily responsible for the protective effect of LPS preincubation: 1) a time dependency of the LPS effect was noted, which is compatible with a gene regulatory event; 2) expression of NOSII protein was noted in LPS-exposed type II cells (Fig. 4); 3) strong up-regulation of NOSII mRNA was directly demonstrated; 4) the LPS-induced increase in the NOSII message demanded the presence of CD14, indicating specific LPS signaling events, and CD14-dependent signal transduction has previously been described to be involved in the endotoxin-induced up-regulation of NOSII in different cell types (46–48); 5) the protective effect of LPS preincubation was abrogated by the NO synthase inhibitor 1-NNMMA, whereas protoporphyrin, used for inhibition of CO generation by hemoxynase, was entirely ineffective; and 6) the protective endotoxin effect was fully reproduced by the administration of two different NO donor agents as well as gaseous NO. The latter effect was particularly impressive, because at the given dosage (250 ppm NO) the gaseous NO itself exerted some epithelial leakage reaction, which was then even reduced upon coapplication with the strong leakage trigger agent H$_2$O$_2$.

The differential sensitivity of the alveolar epithelial cells to apical vs basolateral LPS exposure is a hitherto unreported phenomenon, with underlying reasons demanding further elucidation. A polarized distribution of CD14 and/or associated signal transduction molecules, such as Toll-like receptors, with exclusive presentation to the apical cell surface might offer a ready explanation, but currently no morphological data are available to support such view. Alternatively, a “sensing” of an apical-to-basalversus basolateral-to-apical LPS gradient might represent the underlying mechanisms, as some transepithelial passage of LPS within the 12-h incubation period might be expected for both routes of application. It is tempting to speculate that the alveolar epithelial responsiveness to apically offered endotoxin might be part of an innate defense system, providing protection against bacterial invasion under conditions of pneumonia.

As the most ready explanation for the antipermeability effect of NO, whether derived from endogenous or exogenous sources, its downstream efficacy via guanylate cyclase activation and cGMP increase was considered. In endothelial cell monolayers, elevated cGMP levels were, indeed, demonstrated to protect against a loss of barrier properties provoked by inflammatory agents (49, 50). However, such mode of action was largely excluded for the present NO effect on alveolar epithelial barrier properties. First, LPS preincubation as well as exogenous NO supply did not provide protection against oxidant-unrelated challenges of the alveolar barrier function, as probed with the bacterial exotoxin E. coli hemolysin (being active via induction of phosphatidylinositol response and the provocation of Ca$^+$ $\pm$ fluxes (50, 51)) and the polycation protamine (being operative via its positive charge (52)). Second, any efforts either to increase the intracellular cGMP content in ATII cells (direct application of 8-Br-cGMP; PDE V inhibition for blockage of cGMP metabolism) or to decrease epithelial cGMP levels (guanylate cyclase inhibition by ODQ) were entirely ineffective.

Thus, the NO effect was considered to proceed via some direct interference with reactive oxygen species, appearing under conditions of epithelial H$_2$O$_2$ challenge. From the literature, we are not aware of any evidence for a direct interaction of NO with H$_2$O$_2$, and the presently undertaken in vitro incubation of the NO donor agent NONOate with H$_2$O$_2$ further supported this view. Interestingly, H$_2$O$_2$ treatment of the epithelial cells elicited readily detectable superoxide generation, as measured by lucigenin chemiluminescence technique, and NO interaction with O$_2^-$ was well established. Indeed, the appearance of O$_2^-$ under conditions of ATII H$_2$O$_2$ challenge was reduced both upon ATII preincubation with LPS and in the presence of NONOate. Moreover, the H$_2$O$_2$-elicited leakage response was further enhanced by inhibition of epithelial SOD activity with TETA and was suppressed by exogenous supply with

| Table II. Manipulation of reactive oxygen species: impact on H$_2$O$_2$-induced permeability$^a$ |
|---|---|
| Cells | Paracellular Permeability (%) |
| Control | 100 |
| +LPS pretreatment | 13.2 $\pm$ 8.7$^*$ |
| +NONOate | 13.4 $\pm$ 11$^*$ |
| +TETA | 117.8 $\pm$ 4.5$^*$ |
| +NBT | 81.6 $\pm$ 2.5$^*$ |
| +SOD | 86.5 $\pm$ 4.9$^*$ |
| +MnTBAP | 49.4 $\pm$ 9.2$^*$ |
| +DMTU | 26.1 $\pm$ 6.1$^*$ |

$^a$ Pretreatment with the SOD inhibitor TETA (100 $\mu$m), SOD (100 $\mu$m), the intracellular SOD analog MnTBAP (50 $\mu$m), the O$_2$ scavenger NBT (1 $\mu$m), and the hydroxylradical scavenger DMTU (400 $\mu$m for 30 min) each was performed and compared to the effect of LPS (10 ng/ml for 12 h) and NONOate (100 $\mu$m for 30 min). The increase in permeability in response to H$_2$O$_2$ (100 $\mu$m for 3 h) was set at 100%. Controls were sham incubated. None of the agents investigated exerted any significant effect on the permeability characteristics of the control cells. Data are means $\pm$ SEM of four independent experiments each.

$^*$ $p < 0.05$, for comparison with untreated controls.

| Table III. O$_2^-$ release in epithelial cells: impact of the LPS pretreatment and NO donor application$^a$ |
|---|---|
| Cells | O$_2^-$ Counts (%) |
| Control | 9915 $\pm$ 9 |
| +LPS | 5220 $\pm$ 19$^*$ |
| +NONOate | 4350 $\pm$ 6$^*$ |
| +MnTBAP | 2250 $\pm$ 16$^*$ |

$^a$ Pretreatment with the intracellular SOD analog MnTBAP (50 $\mu$m for 30 min) and NONOate (100 $\mu$m for 30 min), as well as LPS (10 ng/ml for 12 h), was performed. Means $\pm$ SEM of four independent experiments each are given.

$^*$ $p < 0.05$, for comparison with untreated controls.
SOD and, in particular, the intracellular SOD analog MnTBAP. These observations support the view that \( \mathrm{O}_2^- \) appearing in the sequence of events following epithelial \( \mathrm{H}_2\mathrm{O}_2 \) incubation, might contribute to the induction of the strong leakage response, and that \( \mathrm{O}_2^- \) capturing by NO might interfere with this pathway. This suggestion is of interest against a controversial background, questioning whether NO-\( \mathrm{O}_2^- \) interaction with the appearance of peroxynitrite might enhance or suppress inflammatory sequelae such as loss of barrier properties (53–55). However, a putative interaction of NO with \( \mathrm{O}_2^- \) may not fully explain the protective effect of LPS preincubation and NO donor application in the present study, because the intracellular SOD analog MnTBAP provided the strongest scavenging of \( \mathrm{O}_2^- \) but was clearly less effective than LPS and exogenous NO in the presence of CD14. The putative intermediate generation of the \( \cdot\mathrm{OH} \) radical is indicated.

FIGURE 8. Cartoon of the postulated signal transduction pathways investigated in this study. \( \mathrm{H}_2\mathrm{O}_2 \)-induced epithelial permeability increase is antagonized by LPS via NO up-regulation, thereby protecting the epithelial monolayer. Expression of NOSII is critically dependent on the apical route of LPS application and the presence of CD14. The putative intermediate generation of the \( \cdot\mathrm{OH} \) radical is indicated.

In conclusion, apical but not basolateral LPS preincubation of alveolar epithelial cells provided strong protection against a \( \mathrm{H}_2\mathrm{O}_2 \)-elicited epithelial leakage response, and this was clearly attributable to a marked up-regulation of the epithelial NO synthesis. Radical-radical interactions are suggested as underlying mechanisms of the protective NO effect, whereas no evidence was obtained for a role of the NO-dependent cGMP formation. LPS-elicited induction of epithelial NOSII may be considered part of the lung innate immune system, activated under conditions of inhalative bacterial and endotoxin challenge as well as pneumonia.

Acknowledgments

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