Nox2-dependent glutathionylation of endothelial NOS leads to uncoupled superoxide production and endothelial barrier dysfunction in acute lung injury

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1University of Pittsburgh School of Medicine Department of Medicine, Division of Pulmonary Allergy and Critical Care Medicine, Pittsburgh, Pennsylvania; 2University of Pittsburgh Vascular Medicine Institute, Pittsburgh, Pennsylvania; 3University of Pittsburgh School of Medicine Department of Pharmacology, Pittsburgh, Pennsylvania; 4University of Pittsburgh School of Medicine Department of Surgery, Pittsburgh, Pennsylvania; 5University of Pittsburgh School of Medicine Department of Anesthesiology, Pittsburgh, Pennsylvania

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Wu F, Szczepaniak WS, Shiva S, Liu H, Wang Y, Wang L, Wang Y, Kelley EE, Chen AF, Gladwin MT, McVerry BJ. Nox2-dependent glutathionylation of endothelial NOS leads to uncoupled superoxide production and endothelial barrier dysfunction in acute lung injury. Am J Physiol Lung Cell Mol Physiol 307: L987–L997, 2014. First published October 17, 2014; doi:10.1152/ajplung.00063.2014.—Microvascular barrier integrity is dependent on bioavailable nitric oxide (NO) produced locally by endothelial NO synthase (eNOS). Under conditions of limited substrate or cofactor availability or by enzymatic modification, eNOS may become uncoupled, producing superoxide in lieu of NO. This study was designed to investigate how eNOS-dependent superoxide production contributes to endothelial barrier dysfunction in inflammatory lung injury and its resolution. C57BL/6J mice were challenged with intratracheal LPS. Bronchoalveolar lavage fluid was analyzed for protein accumulation, and lung tissue homogenate was assayed for endothelial NOS content and function. Human lung microvascular endothelial cell (HLMVEC) monolayers were exposed to LPS in vitro, and barrier integrity and superoxide production were measured. Bioppterin species were quantified, and cocommunoprecipitation (Co-IP) assays were performed to identify protein interactions with eNOS that putatively drive uncoupling. Mice exposed to LPS demonstrated eNOS-dependent increased alveolar permeability without evidence for altered canonical NO signaling. LPS-induced superoxide production and permeability in HLMVEC were inhibited by the NO inhibitor nitro-l-arginine methyl ester, eNOS-targeted siRNA, the eNOS cofactor tetrahydrobiopterin, and superoxide dismutase. Co-IP indicated that LPS stimulated the association of eNOS with NADPH oxidase 2 (Nox2), which correlated with augmented eNOS S-glutathionylation both in vitro and in vivo. In vitro, Nox2-specific inhibition prevented LPS-induced eNOS modification and increases in both superoxide production and permeability. These data indicate that eNOS uncoupling contributes to superoxide production and barrier dysfunction in the lung microvasculature after exposure to LPS. Furthermore, the results implicate Nox2-mediated eNOS-S-glutathionylation as a mechanism underlying LPS-induced eNOS uncoupling in the lung microvasculature.

acut respiratory distress syndrome; endothelial nitric oxide synthase uncoupling; endothelial barrier function; nitric oxide; NADPH oxidase

INCREASING AGE IMPARTS GREATER RISK for developing acute respiratory distress syndrome (ARDS) and is associated with worse short- and long-term outcomes (14, 19, 22, 27, 30). Both systemic and pulmonary vascular function are known to deter-
Under conditions of limited substrate, cofactor oxidation (32) or posttranslational thiol modification (12) eNOS has been shown to become uncoupled, whereby, rather than oxidize arginine to produce citrulline and NO, electrons transfer univalently to oxygen to produce superoxide. Endothelial NOS uncoupling contributes to endothelial dysfunction in hypertension and atherosclerosis (53, 56), to vascular dysfunction with aging (7, 33), and has been associated with the development of ventilator-induced lung injury in mice (52). Similarly, tetrahydrobiopterin (BH$_4$) supplementation has been demonstrated to protect rats from organ dysfunction and mortality associated with endotoxia (4, 17), and arginase activity is increased in animal models of sepsis (5). We hypothesized that eNOS uncoupling plays a key pathophysiological role in the evolution of microvascular permeability and vasomotor dysfunction in inflammatory acute lung injury and that ROS formation in the inflamed lung may oxidize BH$_4$ or modify eNOS itself, serving to uncouple eNOS and promote “feed-forward” ROS generation and subsequent endothelial barrier dysfunction.

Nox are a major source of O$_2^-$ in vascular and inflammatory cells. In contrast to inflammatory cells, vascular Nox enzymes display a low-level constitutive activity and respond to stimuli on the order of minutes to hours as opposed to instantaneously (24). Vascular Nox2 plays a role in lung endothelial ROS generation in response to ischemia (2), Nox expression is increased in the lung in response to inflammation (18, 24), and various isoforms have been linked to the development of lung injury (11, 40, 44, 54). Given that Nox2 is temporally expressed (24), and various isoforms have been linked to the development of lung injury (11, 40, 44, 54).

We present data demonstrating that eNOS deficiency is protective against LPS-induced lung permeability edema formation in mice. Exploring the mechanism underlying this protection in vitro, we identified a novel association between Nox2 and eNOS in lung microvascular endothelial cells stimulated with LPS. In doing so, Nox2 promotes direct oxidative posttranslational modification of eNOS by S-glutathionylation and sulfenic acid formation. These eNOS modifications drive ongoing superoxide production and endothelial monolayer barrier dysfunction, which is prevented by eNOS inhibition or silencing, and are present in lung tissue from mice exposed to intratracheal LPS.

**MATERIALS AND METHODS**

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

**Materials.** LPS from Escherichia coli (E. coli) 0111:B4 (L-2630), N$_\omega$-Nitro-L-arginine methyl ester hydrochloride (L-NAME), ascorbate, and superoxide dismutase (SOD) were purchased from Sigma-Aldrich; BH$_4$ and 7,8-dihydro-L-biopterin (BH$_2$) were obtained from Cayman Chemical; Nox2 ds-tat peptide was synthesized at the Tufts University Core Facility (http://www.tufts.edu/peptidessynthesis-f.html) as previously described (43a). Anti-eNOS (sc-654), anti-inducible NOS (iNOS) (sc-8310), and anti-Nox2 (sc-74514) were purchased from Santa Cruz Biotechnology; anti-phospho-eNOS S1177 and T495 were obtained from BD Biosciences (612392 and 612706, respectively); anti-glutathione (GSH) (101-A) and anti-cysteine sulfenic acid (SOH, 07-2139) were obtained from Virogen and EMD Millipore, respectively, and anti-GTP cyclohydrolase 1 (ab69962) and β-actin (ab6276) were obtained from Abcam. Silencing RNA, scramble RNA, and transfection reagent (Oligofectamine) were purchased from Dharmacon.

In *in vivo* protocols. Male C57BL/6J (Jackson Laboratories Bar Harbor) and endothelial NOS deficient (eNOS$^{-/-}$) mice (12 and 24 wk old) were anesthetized with 3% isoflurane and exposed to vehicle or 2–3 mg/kg E. coli endotoxin (E. coli O55:B5, List Biologicals) dissolved in sterile water via supraglottic placement and aspiration. Subsequently, they were allowed to recover from anesthesia and had free access to water and chow for 6 or 24 h in a warmed chamber before death. Mice were killed by intraperitoneal overdose of pentobarbital. The left lung was clamped at the hilum, resected, and immediately flash frozen in liquid nitrogen for storage at −80°C. Bronchoalveolar lavage (BAL) was performed on the right lung with serial instillation of three aliquots (0.6, 0.5, 0.5 ml) of warmed sterile saline + 0.6 mM EDTA. Aliquots were pooled and centrifuged at 13,000 g for 5 min, and supernatant was retrieved and frozen at −80°C for subsequent analysis. BAL fluid protein concentration was determined as a measure of alveolar permeability by standard bicinchoninic acid assay (Fisher Thermo Scientific) as previously reported (37, 42, 49).

**Cell culture techniques.** Human lung microvascular endothelial cells (HLMVEC; PromoCell, Lonza) were grown to confluence in endothelial basic medium-2 (EBM-2; Lonza) supplemented with 10% FBS, human recombinant epidermal growth factor, human recombinant insulin-like growth factor-1, human basic fibroblast growth factor, vascular endothelial growth factor, hydrocortisone, ascorbic acid, heparin, gentamicin, and amphotericin B. For experiments, endothelial cells (passages 5–10) were seeded onto 12-well plates and serum starved (EBM-2 + 0.5% FBS) overnight. The cells were then stimulated with LPS (1–50 µg/ml for 0–6 h) and/or in the presence or absence of various pharmacological agents [L-NAME, 100 µM; peglutated SOD (PEG-SOD), 50 U/ml; BH$_4$, 50 µM; BH$_2$, 50 µM; ascorbate, 500 µM; Nox2ds-tat, 10 µM, Scrmrb-tat 10 µM] in a medium consisting of EBM-2 with 5% FBS containing LPS-binding protein and soluble CD14 (sCD14) to promote LPS action according to Goldblum and colleagues (23).

**NOS activity assay.** Cyclic GMP levels were determined in lung tissue homogenates using ELISA according to kit manufacturer instructions (no. 581021, Cayman Chemical) (39, 51). NO activity was measured in tissue homogenates by quantifying the conversion of [3H]-arginine to citrulline in the presence and absence of L-NAME according to kit manufacturer instructions (no. 781001, Cayman Chemical) (3, 13).

**Bioterin quantification.** BH$_4$ levels were measured by high-performance liquid chromatography with florescence detection, as described previously (50). Briefly, lung tissues or HLMVEC were homogenized in ice-cold extract buffer (50 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, 1 mM EDTA) and centrifuged at 16,000 g for 15 min at 4°C. The supernatant was subjected to protein assay and BH$_4$ determination with endotoxemia (4, 17), and arginase activity is increased in various pharmacological agents [L-NAME, 100 µM; peglutated SOD (PEG-SOD), 50 U/ml; BH$_4$, 50 µM; BH$_2$, 50 µM; ascorbate, 500 µM; Nox2ds-tat, 10 µM, Scrmrb-tat 10 µM] in a medium consisting of EBM-2 with 5% FBS containing LPS-binding protein and soluble CD14 (sCD14) to promote LPS action according to Goldblum and colleagues (23).

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was detected at 450 nm emission wavelength after excitation at 350 nm (RF10AXL; Shimadzu). Tetrahydrobiopterin concentration, expressed as picomoles per milligram of protein, was calculated by subtracting BH$_2$ + B from total biopterin.

**ROS measurement.** Superoxide (O$_2^-$) production was measured by L-012 chemiluminescence assay in lung tissue homogenates from C57BL/6J mice 6 or 24 h after LPS exposure (2–3 mg/kg intratracheally, n = 4). Tissue homogenates were incubated with the luminol derivative L-012 (500 μM) for 15 min. Chemiluminescence was quantified over time using a Biotek Synergy 4 Microplate reader (BioTek). The production of O$_2^-$ was confirmed by the addition of PEG-SOD (200 U/ml), and ROS dependence was determined by supplementing the homogenate mixture with t-NNAME (100 μM) (13).

HLMVEC superoxide production was measured in cell media by SOD-inhibitable dichlorofluorescein (DCF) fluorescence and/or electron paramagnetic resonance (EPR) spectroscopy. Endothelial monolayers following treatment with LPS and/or drugs in 12-well plates were washed with PBS and incubated 30 min with H$_2$DCF diacetate (10 μM, Sigma-Aldrich) with or without SOD (400 U/ml). Cells were washed twice with PBS and incubated with 2 mM calcium in PBS (0.5 ml) for 1 h. Supernatant fluorescence was measured using the Biotek Synergy 4 Microplate (excitation and emission wavelengths 485 and 530 nm, respectively) (26). Superoxide production was determined by calculating the fluorescence difference between comparable wells with and without SOD. For EPR, cells were harvested in the absence of proteases by mechanical dissociation and resuspended at 3 × 10$^6$ cells/ml in Chelex-treated PBS pH 7.4. O$_2^-$ formation was assessed using the EPR spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrroline-hCl (CMH) (200 μM) ± t-NNAME (100 μM) or SOD (400 U/ml) for 10 min at 37°C as previously described (35).

**t-NNAME inhibition of NO production.** t-NNAME inhibitable NO production was determined by calculating the fluorescence difference between comparable wells with and without SOD. For EPR, cells were harvested in the absence of proteases by mechanical dissociation and resuspended at 3 × 10$^6$ cells/ml in Chelex-treated PBS pH 7.4. O$_2^-$ formation was assessed using the EPR spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrroline-hCl (CMH) (200 μM) ± t-NNAME (100 μM) or SOD (400 U/ml) for 10 min at 37°C as previously described (35).

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**Western blot.** Lung tissue from LPS-injured and control mice was homogenized in protein lysis buffer (20 mM Tris-HCl, pH 7.0, 1% NP-40, 137 mM NaCl, 1 mM PMSF, 1 μM peptatin, and 1 μM aprotinin) at 4°C, sonicated on ice, and centrifuged at 13,000 g at 4°C. Supernatant was collected and frozen at −80°C for future analysis. Western blot was performed after SDS-PAGE separation of 30 μg of protein on an 8% Tris-glycine gel and transfer to PVDF membrane. Alternatively, HLMVEC were lysed in Laemmli sample buffer, and proteins were separated in 12% SDS gel and transferred to nitrocellulose membranes. Membranes were blocked for 1 h with 5% nonfat milk, incubated with primary antibody overnight at 4°C followed by secondary antibody incubation with species-appropriate horseradish peroxidase-coupled antibody at room temperature for 1 h. Protein detection and quantification were performed using standard chemiluminescence detection techniques (Kodak In Vivo Imaging System). Membranes were subsequently probed with β-actin antibody for normalization of protein loading.

**Coimmunoprecipitation.** Endothelial cells grown in 10-cm plates were washed with ice-cold PBS and incubated on ice for 15 min with 500 μl lysis buffer (20 mM Tris-HCl, pH 7.0, 1% NP-40, 137 mM NaCl, 1 mM PMSF, 1 μM peptatin, and 1 μM aprotinin). The cells were scrape-harvested, and nuclear and cellular debris were removed by centrifugation for 10 min at 16,000 g at 4°C. Alternatively, in a subset of mice exposed to intratracheal LPS, lung tissue was homogenized in lysis buffer (50 mM Tris-HCl, 0.5% NP-40, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 1 mM PMSF, pH 7.5, and 1× protease inhibitor mixture; Roche), and the concentration of protein was determined by BCA assay (Thermo Scientific).

Cell supernatants or lung tissue homogenates were incubated with primary antibody for 1 h at 4°C, followed by addition of 20 μl protein A–G agarose beads (50% slurry) for 1 h at 4°C. Immunoprecipitates were washed three times with ice-cold lysis buffer, separated by centrifugation for 30 s at 16,000 g, and then heated with Laemmli sample buffer for electrophoresis and Western blot analysis. For detection of GSH conjugated with eNOS, eNOS immunoprecipitates were heated with Laemmli sample buffer without 2-mercaptoethanol. For detection of SOH conjugated with eNOS, the lysis buffer was supplemented with 1 mM dimedone, and the Laemmli sample buffer contained 100 mM maleimide without 2-mercaptoethanol (36).

**Endothelial monolayer permeability.** To determine microvascular barrier integrity, HLMVEC were plated on gelatin-coated culture inserts (3–5 × 10$^5$ cells) in 12-well companion plates and grown to confluence in the growth medium. Monolayers were serum-starved overnight and then stimulated with LPS (1–50 μg/ml) in the presence or absence of various pharmacological agents (t-NNAME, 100 μM; PEG-SOD, 50 U/ml; BH$_4$, 50 μM; BH$_2$, 50 μM; ascorbate, 500 μM; Nox2ds-tat, 10 μM, Scrm-btat 10 μM) for 4 h in EBM-2 containing 5% FBS. Subsequently, FITC-labeled dextran (40 kDa, Sigma) was added to the upper chamber (0.1 mg/ml), and PBS was added to the lower chamber (to prevent the formation of an oncotic pressure gradient) for 30 min. Medium was collected from the lower chamber, and the fluorescence was measured using a BioTek fluorimeter (485-nm excitation, 530-nm emission). The fold change in FITC-dextran fluorescence intensity over controls was used as a measure of monolayer paracellular permeability.

**siRNA.** Endothelial cells were seeded in 12-well plates and allowed to grow for 24 h in antibiotic-free EBM-2 containing 10% serum and supplements. Next, each well was incubated with 80 pmol of eNOS or Nox2 siRNA (small interfering ribonucleic acid) or scramble RNA and 3.15 μl of Oligofectamine reagent in 1 ml of antibiotic-free EBM-2 containing 10% FBS and supplements for 24 h. The medium was then changed to regular growth medium, and the cells were cultured for another 24 h. Transfected cells were seeded to inserts for permeability assay or treated with LPS and/or drugs for experiments as described above.

**Statistical analysis.** Primary statistical analyses were performed by Student’s t-tests or one-way ANOVA where appropriate with post hoc analyses using the Bonferroni test for multiple comparisons.

**RESULTS**

**eNOS contributes to LPS-induced lung injury in aged mice.** To determine the role of eNOS in LPS-induced lung injury in vivo, young (10–12 wk, n = 29) and old (24 wk, n = 10) C57BL/6J and eNOS-deficient (eNOS$^{-/-}$, 23 young and 10 old) mice were exposed to intratracheal LPS (2–3 mg/kg in 100 μl) or vehicle (sterile water, 100 μl) and allowed to recover for 6 h. Subsequently, mice were killed, and BAL was performed for protein assay. Intratracheal LPS precipitated lung microvascular permeability in young mice (88 ± 8 μg/ml vehicle vs. 288 ± 36 μg/ml LPS, P = 0.0001), which was accentuated in aged animals (200 ± 41 μg/ml vehicle vs. 1,014 ± 84 μg/ml LPS, P < 0.0001). Whereas young eNOS$^{-/-}$ mice demonstrated a nonsignificant trend toward reduced alveolar permeability, aged eNOS$^{-/-}$ mice were protected against lung microvascular permeability in response to LPS in vivo [BAL protein following LPS = 208 ± 39 μg/ml in young mice (P =...
Fig. 1. Endothelial nitric oxide synthase (eNOS) deficiency is protective against lung injury induced by intratracheal LPS. Depicted are bronchoalveolar lavage (BAL) fluid protein analyses from young (10–12 wk, n = 9 each, mean ± SE) and old (24 wk, n = 3 each) C57BL/6J or eNOS-deficient (eNOS–/–) mice exposed to intratracheal vehicle (100 μl, open or light shaded bars) or endotoxin (LPS, 2–3 mg/kg in 100 μl sterile water, solid or dark shaded bars) for 6 h. LPS-induced BAL protein accumulation is attenuated by eNOS deficiency attenuated by eNOS deficiency in aged mice (P = 0.002 by 1-way ANOVA).

0.2) and 565 ± 83 μg/ml in old mice (P = 0.002, Fig. 1). Measures of canonical eNOS signaling including eNOS phosphorylation, cGMP levels, and arginine-to-citrulline turnover were not altered in lung tissue from young wild-type mice exposed to LPS compared with vehicle (Fig. 2, A and B, n ≥ 3 each, P = NS). As a surrogate measure of eNOS uncoupling in lung tissue, biopetins were quantified in tissue homogenates (50). Lung tissue BH2 was significantly increased in young mouse lungs after LPS exposure compared with vehicle [11.1 ± 1.0 vs. 15.6 ± 0.9 pmol/mg protein in vehicle and LPS-treated animals, respectively (n ≥ 7, P = 0.005), Fig. 2B], suggesting the possibility that eNOS is uncoupled in mouse lungs following LPS exposure. To determine whether uncoupled eNOS may be a source of ROS in the lung, tissue homogenates were prepared from C57BL/6J mice (n = 8) exposed to LPS (2.5 mg/kg in 100 μl sterile water) for 24 h. BAL protein was increased (2,263 ± 233 μg/ml) 24 h after LPS suggesting significant lung injury at this time point. Lung tissue from mice exposed to LPS demonstrated significant l-NAME-dependent superoxide production by L-012 chemiluminescence (0.29 ± 0.05 vs. 0.17 ± 0.02 O2− Abs U/g protein per minute in the absence or presence of l-NAME, respectively; n = 5, P = 0.05) (Fig. 2C), suggestive of NOS dependent superoxide production in LPS-exposed lungs.

Uncoupled eNOS contributes to endothelial permeability in response to LPS in vitro. To further explore the role of eNOS uncoupling in the regulation of alveolar barrier function in response to an inflammatory stimulus, we shifted to an in vitro model. HLMVEC were exposed to LPS (1–50 μg/ml) for up to 6 h, and then O2− production and monolayer permeability were measured. LPS-induced HLMVEC superoxide production, measured by DCF oxidation, peaked 4 h after stimulation (1.7 ± 0.3-fold increase in response to 1 μg/ml, Fig. 3A), was
Fig. 3. Bacterial LPS induces superoxide production and barrier dysfunction in human lung microvascular endothelial cells (HLMVEC) in vitro. A: superoxide dismutase (SOD)-inhibitable dichlorofluorescein (DCF) fluorescence in media extracted from cultured HLMVEC following bacterial LPS exposure (0–50 μg/ml) for 0–6 h in vitro. B: fluorescent dextran accumulation in basal media after apical exposure of HLMVEC to bacterial LPS (1 μg/ml) for 0–6 h in vitro. LPS induces dose-independent superoxide production and increased permeability to fluorescent dextran peaking at 4 h after stimulation. Bars represent the means ± SE of n = 3 separate experiments for each dose and at each time point. †P < 0.0001 and *P < 0.01 by 1-sided paired t-test. 1-way ANOVA used for comparison of dose or time dependence. RFU, relative fluorescence units. C: HLMVEC exposed to LPS (50 μg/ml) ± pegylated SOD (PEG-SOD) (25 U/ml) for 12 h, harvested in the absence of proteases by mechanical dissociation, and resuspended at 3 × 10⁶ cells/ml in Chelex-treated PBS pH 7.4. Superoxide formation was monitored with the electron paramagnetic resonance (EPR) spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine·HCl (CMH) (200 μM) ± L-NAME (100 μM) for 10 min at 37°C. Spectra represent CM intensity consisting of 5 signal-averaged scans from t = 9 to t = 10 min. Tracings displayed from top to bottom are as follows: untreated, LPS treated, LPS treated (+PEG-SOD), LPS treated (+i-NAME). LPS increased the CMH EPR signature amplitude by 65% over untreated cells. Both i-NAME and SOD attenuated the signal to levels comparable to untreated cells.

Fig. 4. Bacterial LPS-induced superoxide production is associated with the development of barrier dysfunction in vitro. A: SOD-inhibitable DCF fluorescence in media extracted from HLMVEC following LPS exposure (1 μg/ml for 4 h) in the presence of ascorbate (500 μM) (Asc) or BH₄ (50 μM). B: fluorescent dextran accumulation in basal media after apical exposure of HLMVEC to LPS (1 μg/ml for 4 h) in the presence of ascorbate (500 μM), BH₄ (50 μM), or SOD (50 U/ml). SOD experiment is shown separately, as it represents separate experiment with its own control cells. LPS-induced permeability is attenuated in the presence of antioxidants or superoxide-specific SOD. Bars represent means ± SE of n = 3 separate experiments for each dose and for permeability independent of dose, and correlated with an increase in monolayer dextran permeability (1.6 ± 0.1-fold increase after 4 h compared with baseline, Fig. 3B). O₂⁻ production after high-dose LPS treatment (50 μg/ml) was confirmed by EPR using the cyclic hydroxylamine CMH, which demonstrated a 65% increase in signal over untreated HLMVEC (Fig. 3C, top) and correlated with increased monolayer permeability (data not shown). LPS-induced (1 μg/ml) superoxide production and dextran permeability were attenuated by administration of the nonspecific antioxidants ascorbate and BH₄ (Fig. 4, A and B), both of which may serve to recouple eNOS function.

Canonical eNOS signaling was assessed in HLMVEC exposed to LPS for 4 h. Interestingly, iNOS was not detectable in cultured HLMVEC (Fig. 5A). Neither iNOS nor eNOS protein expression was altered in HLMVEC in response to LPS stimulation. Similarly, canonical eNOS phosphorylation was unchanged in LPS-exposed HLMVEC (Fig. 5A). Measurement of NO production showed a significant decrease in HLMVEC 4 h after LPS exposure to levels similar to those of eNOS-silenced cells (Fig. 5, B and C).
To determine whether uncoupled eNOS was in part responsible for LPS-induced $\text{O}_2^-$ production, $\text{H}_2\text{O}_2$ DCF oxidation to DCF was measured after NO inhibition. Both pharmacological inhibition of NOS by l-NAME (100 μM) and eNOS silencing prevented HLMVEC superoxide production induced by low-dose LPS (1 μg/ml) stimulation (l-NAME + LPS 1.2 ± 0.3-fold vs. LPS 1.7 ± 0.4-fold, eNOS siRNA + LPS 0.9 ± 0.1-fold vs. eNOS scrRNA + LPS 1.8 ± 0.2-fold compared with baseline, Fig. 5C). Consistent with this finding, both l-NAME and SOD attenuated the CM EPR signal induced by high-dose LPS (50 μg/ml) to levels comparable to untreated HLMVEC (Fig. 3C, middle bottom and bottom). Taken together, these data suggest that eNOS provides a source for superoxide production in HLMVEC stimulated with LPS.

Endothelial monolayer permeability increased ~1.7-fold following LPS stimulation (1 μg/ml for 4 h). Similar to the effect on superoxide production, treatment with l-NAME (100 μM) abrogated the permeability increase (1.2 ± 0.1-fold increase compared with baseline control, Fig. 5E). Similar to results published by Predescu and colleagues (43), eNOS silencing increased basal permeability in HLMVEC monolayers (1.6 ± 0.1-fold increase compared with eNOS scramble RNA control), suggesting a role for endogenous NO production in the maintenance of barrier integrity. Confirming the effects of l-NAME, eNOS silencing also abrogated the increase in monolayer permeability induced by LPS in vitro (63 ± 13% increase after LPS in scramble RNA controls vs. 14 ± 11% increase after LPS in silencing RNA experiments, $P = 0.04$ for difference in change, Fig. 5E). These data suggest that eNOS uncoupling contributes to LPS-induced monolayer barrier dysfunction in HLMVEC.

Nox2 contributes to endothelial permeability in response to LPS in vitro. As previously indicated, vascular Nox2 plays a role in lung endothelial ROS generation in response to ischemia (2), and increased Nox expression has been described in the lung in response to inflammation (18, 24). In our model system, endothelial Nox2 expression was increased following LPS exposure (Fig. 6A). As with eNOS silencing, Nox2 silencing slightly increased basal permeability in HLMVEC monolayers (1.2 ± 0.1-fold increase compared with Nox2 scramble RNA control, $P = 0.1$). Inhibition of Nox2 using the specific peptide inhibitor (Nox2ds-tat) and silencing of Nox2 using a specific siRNA strategy (Fig. 6A) prevented both LPS-induced superoxide production [LPS + Scrmb-tat 1.7 ± 0.1-fold increase vs. LPS + Nox2ds-tat 1.2 ± 0.1-fold increase ($P = 0.005$)], LPS + Nox2 scramble RNA 1.5 ± 0.1-fold increase vs. LPS + Nox2 scramble RNA 1.1 ± 0.1-fold increase ($P = 0.0006$), Fig. 6B] and monolayer permeability (48 ± 11% increase after LPS in
scramble RNA controls vs. 5 ± 7% increase in silencing RNA experiments, \( P = 0.004 \) for difference in change, Fig. 6C) in vitro. These data suggest that, in addition to eNOS uncoupling, Nox2-dependent superoxide production contributes to LPS-induced monolayer barrier dysfunction in HLMVEC.

Nox2-dependent oxidation of eNOS drives uncoupling in LPS-stimulated lung endothelium. As noted previously, bioppterin oxidation or oxidative modification of the enzyme directly may lead to the uncoupling of eNOS enzymatic function and the production of \( \text{O}_2^- \) in lieu of NO. Our in vivo data suggested that bioppterin oxidation contributed to eNOS uncoupling in the inflammatory lung injury (Fig. 2B). Given that Nox2 contributes to superoxide production in HLMVEC exposed to LPS, we explored the possibility that bioppterin oxidation leads to subsequent eNOS uncoupling in LPS-exposed cells. In contrast to LPS-exposed mouse lungs, bioppterin levels were unchanged in HLMVEC treated with LPS, and BH2 supplementation did not affect monolayer barrier integrity (dextran increase from baseline in LPS 1.5 ± 0.1-fold vs. LPS + BH2 1.8 ± 0.4-fold, \( P = \text{NS} \), Fig. 7), suggesting that this is not the mechanism underlying eNOS uncoupling in lung microvascular endothelial cells.

We next explored the possibility that Nox2-dependent superoxide production modifies the eNOS protein leading downstream to uncoupling of the enzyme. Coimmunoprecipitation revealed that eNOS associates with Nox2 in HLMVEC following LPS stimulation (Fig. 8A). Similarly, LPS induces S-glutathionylation of eNOS protein (eNOS-GSH) and eNOS cysteine oxidation to sulfenic acid (eNOS-SOH) that is inhibited in the presence of the Nox2ds-tat peptide inhibitor (Fig. 8, B and C). Taken together, these observations suggest that Nox2-dependent superoxide production leads to posttranslational oxidative modifications of the eNOS protein that promote uncoupling of enzymatic function and eNOS-dependent superoxide production.

Finally, we returned to the murine model to determine whether Nox2 associates with eNOS and induces S-glutathionylation of eNOS protein (eNOS-GSH) in lungs exposed to LPS in an age-dependent manner. Coimmunoprecipitation of mouse lung tissue homogenates 6 h following intratracheal LPS administration reveals that eNOS does in fact associate with Nox2 in the injured lung and that eNOS protein is glutathionylated to a greater degree in aged mice compared with young mice (Fig. 9). Furthermore, Nox2 association with eNOS and subsequent S-glutathionylation are associated with a nonsignificant trend toward increased \( \text{O}_2^- \) production in old mice compared with young mice as determined by L-012 chemiluminescence [0.36 ± 0.02 (aged) vs. 0.29 ± 0.02 (young) Abs/mg lung tissue per min, \( n = 4 \), \( P = 0.07 \) by two-tailed \( t \)-test, data not shown] without significant difference in the L-NAME-dependent component.
DISCUSSION

Pulmonary endothelial barrier integrity is dependent on local NO bioavailability (43), which also serves to regulate local inflammation, reducing leukocyte and platelet adhesion to the endothelium. eNOS is the key enzyme regulating local NO production at the level of the endothelium, but the role eNOS plays in the development of lung injury remains unclear. When uncoupled, eNOS produces superoxide in lieu of NO (56), thereby reducing NO bioavailability and producing a potentially toxic product known to contribute to endothelial cytoskeletal reorganization and paracellular gap formation (28), both key mechanisms underlying the development of endothelial permeability (21, 46). eNOS uncoupling has recently been associated with the development of lung injury in the setting of high tidal volume mechanical ventilation (52). Our data both in vivo and in vitro support the hypothesis that eNOS uncoupling contributes to lung microvascular endothelial barrier dysfunction in the setting of inflammatory lung injury. The mechanisms by which \( \text{O}_2^- \) contributes to lung endothelial permeability, however, remain unanswered by this work and represent one focus of ongoing work in the laboratory.

From a mechanistic perspective, our study provides novel insight into how eNOS may become uncoupled in inflammatory states. Three potential mechanisms have been demonstrated to uncouple NOS: 1) substrate limitation, 2) oxidation of the necessary cofactor, BH4 (32), and 3) posttranslational oxidative modification of the enzyme (12). Nox are important sources of superoxide production in inflammatory cells as well as the microvasculature of the lung (2, 11, 18, 24, 25, 40, 44, 54), and deficiency of Nox2 has been demonstrated to be protective against lung edema formation in a murine model of Gram-negative sepsis (20). We hypothesized that Nox2-dependent oxidative modification of eNOS protein \( S\)-glutathionylation and sulfenic acid formation is dependent on Nox2.
dent superoxide production and/or downstream reactive metabolites would contribute to lung microvascular endothelial barrier dysfunction in response to LPS through oxidation of BH4 or the eNOS enzyme itself. Indeed, our data demonstrate that specific inhibition of Nox2 reduces superoxide production by lung microvascular endothelial cells and prevents the barrier disruption that follows endotoxin exposure in vitro (Fig. 6).

Our data in vivo suggest that BH4 oxidation may contribute to NO uncoupling in inflammatory lung injury with BH2 being increased in lung tissue from mice exposed to intratracheal LPS (Fig. 2). However, our in vitro experiments indicate that the source of biopterin in the intact lung is not the microvascular endothelial cells. Neither BH4 nor BH2 were detectable in HLMVEC lysates in the presence or absence of LPS (Fig. 7), and treatment of the endothelium with BH2 did not contribute to barrier disruption in vitro. Taken together, these data suggest that this mechanism is not particularly important for regulation of eNOS function in the lung microvasculature. It is possible that conduit endothelium, inflammatory cells, or epithelial cells contribute to the biopterin pool in the intact lung. Further study is required to elucidate the source of biopterin in the lung and the contribution of biopterin oxidation to NO uncoupling in vivo.

As oxidative modification of eNOS, e.g., -glutathionylation, promotes uncoupling of enzymatic function (12), we explored the hypothesis that the barrier protection afforded by Nox2 inhibition was potentially mediated through regulation of eNOS oxidation. Our data demonstrate that eNOS protein associates with Nox2 in lung microvascular endothelium after exposure to LPS. This close approximation may enable low-level Nox2-catalyzed superoxide and/or reactive metabolites of superoxide to modify the structure of eNOS, thereby driving uncoupling and accentuating superoxide production (Fig. 10). To answer this question, we examined whether LPS exposure results in oxidative modification of the eNOS protein. Our data clearly show that LPS causes both -glutathionylation of the eNOS protein as well as eNOS SOH formation in a Nox2-dependent fashion in vitro (Fig. 8). Furthermore, our data suggest that oxidative modification of eNOS occurs in vivo in response to endotoxin exposure in an age-dependent manner (Fig. 9). Although this particular experiment was underpowered to detect a significant difference in $O_2^\cdot$ production in vivo, aged mice exhibited a trend toward increased ROS production after LPS. Further study is required to validate 1) age-dependent differences in $O_2^\cdot$ production, 2) define specific enzymatic source(s) in this model, and 3) identify factors that may alter the identity of downstream metabolites of $O_2^\cdot$ requisite for oxidative modification eNOS. The association of Nox2 with eNOS following inflammatory stimulation represents a previously undescribed mechanism underlying barrier dysregulation in the lung microvascular endothelium, and this mechanism of cross talk between redox enzymes to promote uncoupling of eNOS has not previously been described in the literature. How these enzymes come together at the cell surface remains yet to be explored although both enzymes have been previously associated with caveolae (38).

The population admitted to intensive care units across the country and around the world is aging (22). From a translational perspective, NO uncoupling has been associated with vascular dysfunction with aging (7, 29, 33), and it is well described that vascular endothelial dysfunction plays a key role in the development and consequences of ARDS (10, 21, 37, 42, 45). Furthermore, the risk of developing ARDS increases with age, and both short- and long-term outcomes are worse in elderly patients (14, 19, 22, 27, 30). Similar to data in humans, our data suggest that older mice develop worse lung injury in response to LPS compared with their younger counterparts, and deficiency of eNOS is protective against lung edema formation in aged mice (Fig. 1). Taken together, these data suggest a potentially important role for eNOS uncoupling in the development of barrier dysfunction with age. One potential explanation for these phenomena is that NOS becomes uncoupled with age, reducing NO bioavailability and predisposing to lung microvascular barrier dysfunction at baseline, which is then exacerbated with inflammatory stimulation. Although we have demonstrated that eNOS associates with Nox2 and undergos -glutathionylation in an age-dependent manner after stimulation with LPS in vivo (Fig. 9), more work is necessary to further validate our findings in vivo and to determine the time course and relative importance of age-dependent NO uncoupling to the development of lung injury. As antioxidant therapies have not proven successful in treating lung injury in all work to date, further work is necessary to explore the therapeutic potential of strategies aimed at recoupling NO function and restoring NO bioavailability for the treatment of inflammatory lung injury in patients demonstrating a vascular phenotype of disease. Perhaps these strategies may prove beneficial in aging populations.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.
AUTHOR CONTRIBUTIONS


REFERENCES


