Lipopolysaccharide (LPS)-mediated Angiopoietin-2-dependent Autocrine Angiogenesis Is Regulated by NADPH Oxidase 2 (Nox2) in Human Pulmonary Microvascular Endothelial Cells*

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Background: The mechanisms by which bacterial ligands alter angiogenesis remain unknown. Results: Lipopolysaccharide-mediated Angiopoietin-2-dependent autocrine angiogenesis in lung endothelial cells is regulated by NADPH oxidase 2. Conclusion: Endothelial Nox2 regulates Angiopoietin-2-dependent angiogenesis. Significance: This study presents new data regarding the regulation of proinflammatory angiogenesis.

Sepsis-mediated endothelial Angiopoietin-2 (Ang2) signaling may contribute to microvascular remodeling in the developing lung. The mechanisms by which bacterial cell wall components such as LPS mediate Ang2 signaling in human pulmonary microvascular endothelial cells (HPMECs) remain understudied. In HPMEC, LPS-induced Ang2, Tie2, and VEGF-A protein expression was preceded by increased superoxide formation. NADPH oxidase 2 (Nox2) inhibition, but not Nox4 or Nox1 inhibition, attenuated LPS-induced superoxide formation and Ang2, Tie2, and VEGF-A expression. Nox2 silencing, but not Nox4 or Nox1 silencing, inhibited LPS-mediated inhibitor of κ-B kinase β (IKKβ) and p38 phosphorylation and nuclear translocation of NF-κB and AP-1. In HPMECs, LPS increased the number of angiogenic tube and network formations in Matrigel by >3-fold. Conditioned media from LPS-treated cells also induced angiogenic tube and network formation in the presence of Toll-like receptor 4 blockade but not in the presence of Ang2 and VEGF blockade. Nox2 inhibition or conditioned media from Nox2-silenced cells attenuated LPS-induced tube formation and network formation. Ang2 and VEGF-A treatment rescued angiogenesis in Nox2-silenced cells. We propose that Nox2 regulates LPS-mediated Ang2-dependent autocrine angiogenesis in HPMECs through the IKKβ/NF-κB and MAPK/AP-1 pathways.

Vascular remodeling in bronchopulmonary dysplasia (BPD), characterized histologically by a paucity of blood vessels and dysmorphic arborization in the distal lung, are hallmark marks of the disease in the post-surfactant era (1, 2). With increasing use of non-invasive ventilation and controlled use of supplemental oxygen, the contribution of chorioamnionitis and bacterial sepsis to pulmonary vascular injury in premature infants has become more evident (3–5). Gram-negative bacterial cell wall components such as LPS released during sepsis induce a change in the endothelial phenotype from a quiescent phenotype to a “proinflammatory” phenotype (6, 7). This switch is associated with increased Angiopoietin-2 (Ang2) expression and disruption of homeostatic Angiopoietin 1 (Ang1)/Tie2 signaling, resulting in endothelial expression of cellular adhesion molecules and cytokines that facilitate lung inflammation and injury (1, 6–8). Furthermore, Ang2 and Ang1 expression is regulated temporally in the developing lung and facilitates development of the pulmonary vascular network (1, 9). Given the importance of Ang2 signaling in neonatal lung injury and pulmonary vascular development, elucidating the mechanisms underlying bacteria-mediated Ang2 expression in the pulmonary endothelium will contribute to a better understanding of the pathogenesis of BPD and assumes translational significance (9, 10).

In endothelial cells, Ang1 secreted from pericytes, smooth muscle cells, and fibroblasts binds to Tie2 (endothelial cell surface tyrosine kinase receptor), inducing tyrosine autophosphorylation (6, 7). Constitutive Ang1/Tie2 signaling promotes endothelial survival and migration and antagonizes the proinflammatory effects of cytokines and VEGF in the mature endothelium (7, 11). Ang2 competes with Ang1 for Tie2 and promotes the expression of cellular adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1), increases vascular permeability, and augments leukocyte transmigration (7, 10, 11). The effects of Ang2 on angiogenesis are modulated by the presence or absence of VEGF. In the presence of VEGF, Ang2 induces migration, proliferation, and the sprouting of new blood vessels, whereas, in the absence of VEGF, endothelial cell...
death and vessel regression occur (7, 11). Although systemic LPS administration has been shown to augment Ang2 expression in the liver, lung, and other tissues, the mechanisms involved in LPS-mediated Ang2 expression in lung endothelial cells remain unknown (12). Specifically, the involvement of redox signaling in the regulation of Ang2-dependent signaling and endothelial cell angiogenesis has not been studied.

NADPH oxidases (Nox) have been reported to regulate endothelial responses to bacterial ligands and proinflammatory stimuli (13–15). Nox enzymes belong to a family of multimeric proteins that catalyze one electron reduction of oxygen to generate superoxide using NADPH as substrate (14). Nox-dependent redox signaling serves key physiologic processes in the endothelium but is also implicated in pathological processes such as ischemia-reperfusion, inflammation, and cell death (14, 16). Nox2, Nox4, and Nox1 have all been shown previously to mediate cytokine expression in response to LPS in endothelial cells of varied lineages (13, 15, 17). In this study, we investigated the hypothesis that Nox regulates LPS-mediated Ang2 signaling and angiogenesis in human pulmonary microvascular endothelial cells (HPMECs). Here we demonstrate that Nox2 regulates LPS-mediated Ang2 and VEGF-A expression in HPMECs through the IKKb/NF-kB and p38/AP-1 pathways. We also show that Ang2- and VEGF-A-mediated autocrine angiogenesis is regulated by Nox2 in lung endothelial cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents—**Fetal HPMECs (ScienCell, Carlsbad, CA) were used between passages 3–5 for all experiments. HPMECs were grown in endothelial cell medium (ECM) supplemented with fetal bovine serum, antibiotics, and endothelial cell growth serum as recommended by the manufacturer (ScienCell) in a humidified incubator containing 5% CO2 at 37 °C. Ultrapure LPS (100 ng/ml) and human TLR4 neutralizing antibody (Ab-TLR4, 5 μM) were purchased from Invivogen (San Diego, CA). Tiron, potassium phosphate, EGTA, sucrose, and Nox4 were purchased commercially from Santa Cruz Biotechnology (Santa Cruz, CA), and cDNA was synthesized from 1 μg of RNA using the iScript cDNA synthesis kit (Bio-Rad) according to the instructions of the manufacturer. The transcripts were amplified, and gene expression data were collected on a Bio-Rad IQ5 with SYBR Green Mastermix. In experiments using PEG-superoxide dismutase and VAS2870, the chemicals were incubated with the cells for 1 h prior to LPS treatment. The primers for Ang2, VEGF-A, and Tie2 were obtained from Operon (Huntsville, AL). They consisted of Ang2 (sense, GAGAAGCTGTCTCGAAGCT; antisense, GTGGAAGAGGACAAGTGT), VEGF-A (sense, GGCGAGAACATCATCGAGGA; antisense, ATCTGTGATGGTGTGTTGAA), and Tie2 (sense, TACTAAATGAAAGATGACCTTGG; antisense, GGAGGTGTGTAATGTTGAAATCT). The primers for Nox1, Nox2, and Nox4 were purchased commercially from Santa Cruz Biotechnology (Santa Cruz, CA), and the primers for 18 S were purchased commercially from MCLabs (San Francisco, CA). 18 S was used as the housekeeping gene. Relative gene expression of Nox1, Nox2, Nox4, Ang2, VEGF-A, and Tie2 was calculated with the Pfaffl method (18).

**Immunoblotting for Quantifying Changes in Protein Expression and Phosphorylation—**Whole cell lysates were prepared from HPMECs by lysing cells with a modified radioimmune precipitation assay buffer containing commercially available protease and phosphatase inhibitors (Sigma). Protein quantification was done using a BCA protein assay from Thermo Fisher (Rockford, IL) according to the instructions of the manufacturer using BSA as a standard. Immunoblotting was done following standard protocol. The primary antibodies for angiogenic expression were as follows: mouse anti-Tie2 (Cell Signaling Technology, Danvers, MA, 1:1000), rabbit anti-Ang2 (Abcam, Cambridge, MA, 1:1000), rabbit anti-VEGF-A (Santa Cruz Biotechnology, 1:500), and mouse anti-β-Actin (Sigma, 1:5000). For phosphorylation, the primary antibodies were as follows: rabbit anti-(p)IKKβ, rabbit anti-(p)38 MAPK, rabbit anti-(p)SAPK/JNK, rabbit anti-p38 MAPK, rabbit anti-SAPK/JNK (Cell Signaling Technology, 1:1000), and mouse anti-IKKβ.
reaction mixture was added to each well, containing the following components: NADPH, lucigenin, and 50 mm potassium phosphate buffer with 1 mM EGTA. The cells were treated with LPS for 7–45 min. Each condition was run in quintuplicate, the Tiron-inhibition (5 µM) mixture, the chemiluminescent signals were collected every 1 min for 30 min in a 96-well plate, with the total relative light units summated for each well at the end of 10 min (luminometry). The cell lysates were prepared using a Tiron-inhibitable lucigenin chemiluminescence assay as described previously (19, 20). Briefly, 1–2 × 10^4 cells were seeded in a white 96-well plate (Bio-Rad) and grown for 24 h. Cells were treated with LPS (100 ng/ml) for 7–45 min. Each condition was run in quintuplicate, the Tiron-inhibition (5 µM) wells were run in duplicate, and a blank well was run with only lucigenin. After LPS incubation, cells were washed briefly with Heps-buffered Saline twice. After the last wash, 100 µl of 50 µM potassium phosphate buffer with 1 mM EGTA and 1:1000 protease inhibitor mixture (Sigma) was added to each well. Immediately after addition of the phosphate buffer, a lucigenin reaction mixture was added to each well, containing the following reaction components (final concentration): NADPH (100 µM), sucrose (150 mM), and lucigenin (5 µM). After the lucigenin mixture, the chemiluminescent signals were collected every 1 min for 30 min in a 96-well plate, with the total relative light units summated for each well at the end of 10 min (luminometry from Turner Scientific, Madison, WI). The summated signal inhibited by Tiron was calculated and subtracted from each corresponding sample. Superoxide anion results are expressed as Tiron-inhibited NADPH oxidase activity detected by chemiluminescent signals (relative light units).

siRNA-mediated Nox1, Nox2, and Nox4 Gene Silencing—siRNA sequences targeting Nox1, Nox2, and Nox4 were purchased from Santa Cruz Biotechnology, and transfections were performed as before (13). Briefly, cells were cultured with antibiotic-free ECM until 60–80% confluent. The media was then aspirated, and cells were washed twice with siRNA transfection medium (Santa Cruz Biotechnology). The plates were then incubated with either the control or siRNA strand (1 µg) in transfection medium and incubated for 16 h. Subsequently, the reagents were aspirated, and normal ECM was gently put on the plates. The optimal period of silencing was determined as 36 h for Nox1 and 48 h for Nox2 and Nox4 using mRNA and protein studies. The silencing efficiency was determined by PCR (primers mentioned above) and by immunoblotting using goat anti-Nox1 (1:500), goat anti-Nox2 (1:500), and rabbit anti-Nox4 (1:500) antibodies purchased from Santa Cruz Biotechnology. β-Actin was used for normalization.

Immunoprecipitation of Nox2—Cells grown to 90% confluence in 50-mm dishes were treated with LPS for 15 or 30 min or left untreated. The beads were prepared with goat anti-gp91phox antibody (Santa Cruz Biotechnology) according to the protocol of the manufacturer using SureBeads™ protein G magnetic beads for immunoprecipitation (Bio-Rad). A sample (500 µg) of the protein was incubated with the magnetic antibody beads overnight at 4 °C. Upon completion, the beads were magnetized and washed twice with ice-cold TBS, after which 50 µl of 2× Laemmlı buffer was incubated with the beads and heated at 95 °C prior to immunoblotting. Blots were incubated overnight at 4 °C with goat anti-gp91phox (Santa Cruz Biotechnology, 1:500) and rabbit anti-p47phox (Santa Cruz Biotechnology, 1:500). Blots were developed using ECL, and densitometry was performed using ImageJ software (National Institutes of Health).

Detection of NADPH-dependent Superoxide Formation—NADPH-dependent production of superoxide was quantified using a Tiron-inhibitable lucigenin chemiluminescence assay as described previously (19, 20). Briefly, 1–2 × 10^4 cells were seeded in a white 96-well plate (Bio-Rad) and grown for 24 h. Cells were treated with LPS (100 ng/ml) for 7–45 min. Each condition was run in quintuplicate, the Tiron-inhibition (5 µM) wells were run in duplicate, and a blank well was run with only lucigenin. After LPS incubation, cells were washed briefly with Heps-buffered Saline twice. After the last wash, 100 µl of 50 µM potassium phosphate buffer with 1 mM EGTA and 1:1000 protease inhibitor mixture (Sigma) was added to each well. Immediately after addition of the phosphate buffer, a lucigenin reaction mixture was added to each well, containing the following reaction components (final concentration): NADPH (100 µM), sucrose (150 mM), and lucigenin (5 µM). After the lucigenin mixture, the chemiluminescent signals were collected every 1 min for 30 min in a 96-well plate, with the total relative light units summated for each well at the end of 10 min (luminometry from Turner Scientific, Madison, WI). The summated signal inhibited by Tiron was calculated and subtracted from each corresponding sample. Superoxide anion results are expressed as Tiron-inhibited NADPH oxidase activity detected by chemiluminescent signals (relative light units).

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FIGURE 1. LPS induces the expression of angiogenic markers and superoxide in HPMECs. A, Ang2, VEGF-A, and Tie2 mRNA expression quantified by real-time PCR 3, 7, and 24 h after LPS treatment (n = 4). *, p < 0.02 (Ang2, control versus 3, 7, and 24 h LPS); **, p < 0.02 (VEGF-A, control versus 3, 7, and 24 h LPS); ***, p < 0.01 (Tie2, control versus 3 and 7 h LPS). B, cell lysates obtained from LPS-treated or control HPMECs at 12, 24, and 36 h were immunoblotted for Tie2, Ang2, and VEGF-A. C, densitometry quantification of angiogenic markers. *, p < 0.007 (12 h Tie2, control versus LPS); #, p = 0.003 (12 h Ang2, control versus LPS); $, p < 0.01 (the rest of the comparisons are between control versus LPS for the respective proteins) (n = 4). D, Ang2 and VEGF-A protein expression was quantified in cell lysates by immunoblotting 24 h after treatment with LPS or LPS + Ab-TLR4. E, densitometric quantification of Ang2 and VEGF-A following treatment with LPS and Ab-TLR4, *, p < 0.01 (Ang2, control versus LPS, LPS versus 2.5 μM, and LPS versus 5 μM); $, p < 0.03 (VEGF, control versus LPS, LPS versus 2.5 μM, and LPS versus 5 μM) (n = 3); F, superoxide formation in HPMECs was quantified at various time points after LPS treatment using a lucigenin-derived chemiluminescence assay. *, p = 0.02 (control versus 7 min LPS); **, p < 0.001 (control versus 15 min LPS); ***, p < 0.02 (control versus 30 min LPS); $, p = 0.04 (control versus 45 min LPS) (n = 3). G, lucigenin chemiluminescence was quantified at 1-min intervals in control and LPS-treated (15 min) HPMECs as before. *, p < 0.05 (control versus LPS for all comparisons, n = 3). H, Ang2, VEGF-A, and Tie2 RNA expression quantified by real-time PCR 3 h after LPS treatment. *, p < 0.02 (Ang2, control versus 3 h LPS, LPS versus LPS + PEG-superoxide dismutase); **, p < 0.02 (VEGF-A, control versus 3 h LPS, LPS versus LPS + PEG-superoxide dismutase); ***, p < 0.01 (Tie2, control versus 3 h LPS, LPS versus LPS + PEG-superoxide dismutase, and LPS versus LPS + PEG-superoxide dismutase) (n = 3). Cells were pretreated with VAS2870 and PEG-superoxide dismutase for 1 h before LPS.
with Ab-Ang2/VEGF (blocking antibody), HPMEC were treated with 500 ng/ml of antibody for 30 min before addition of CM.

**Statistical Analysis**—Statistical analysis was done using STATA 12 (StataCorp LP, Dallas, TX). Data are presented as mean ± S.D. \( p < 0.05 \) was considered significant for experiments. Fold changes in protein levels relative to the untreated control cells were quantified by densitometry and compared between various treatments using analysis of variance. Changes in superoxide levels, transcription factor levels, and angiogenic formations relative to the untreated, control cells were compared between using analysis of variance. The Bonferroni test was used in conjunction with analysis of variance to perform pairwise comparisons between groups. For mRNA studies, changes in gene expression with various treatments were calculated relative to expression in control cells and compared between different treatment groups using analysis of variance.

**RESULTS**

**LPS-induced Ang2, Tie2, and VEGF-A Expression in HPMECs Is Associated with Increased Oxidative Stress**—The effect of LPS (100 ng/ml) on Ang2, Tie2, and VEGF-A RNA and protein expression were studied in HPMECs. LPS-induced Ang2, Tie2, and VEGF-A RNA expression was evident at 3 h, peaked by 7 h, and decreased by 24 h (Fig. 1A). Correspondingly, LPS-mediated expression of Ang2, Tie2, and VEGF-A protein was detectable by 12 h, peaked at 24 h, and decreased by 36 h (Fig. 1, B and C). TLR4 (an LPS recognition receptor) blockade using a specific antibody resulted in a dose-dependent inhibition of Ang2 and VEGF-A expression (Fig. 1, D and E). These data show that LPS mediates TLR4-dependent expression of angiogenic markers in HPMECs.

To assess the effect of LPS on oxidative stress in HPMECs, we measured NADPH-dependent superoxide formation. Superoxide formation represents a summation of data collected every minute over 10 min after timed LPS treatments. LPS caused a temporal increase in superoxide formation, which was detectable by 7 min, peaked at 15 min, and persisted at 45 min (Fig. 1F). The time course of superoxide formation after LPS treatment for 15 min is shown in Fig. 1G. To demonstrate the relevance of LPS-induced superoxide to the expression of angiogenic markers, we conducted experiments with VAS2870 (a NOX inhibitor) and PEG-superoxide dismutase (a superoxide anion scavenger) (21, 22). Pretreatment with VAS2870 (10 \( \mu \)M) or PEG-superoxide dismutase (400 units/ml) for 1 h attenuated LPS-mediated induction of Ang2, VEGF-A, and Tie2 RNA at 3 h (Fig. 1H). These data suggest that LPS-mediated superoxide formation is important for induction of angiogenic markers.

**LPS Induces Lung Endothelial Expression of Tie2 and Ang2 in Vivo**—To investigate whether changes in Tie2 expression observed in vitro with LPS in HPMEC are observed in vivo, we isolated mouse lung endothelial cells 18 h after intraperitoneal LPS (1 mg/kg) injection in 7-day-old mice. Mouse lung endothelial cells showed a >2.2-fold increase in Tie2 and Ang2 expression after systemic LPS (Fig. 2, A and B). These data demonstrate that LPS induces Tie2 protein in lung endothelial cells in neonatal mice.

**LPS Stimulates Angiogenic Tube and Network Formation in HPMECs in a VEGF-A/Ang2-dependent Manner**—We then examined the effect of LPS on in vitro HPMEC angiogenesis in a Matrigel-based assay used by other investigators (23). LPS increased angiogenic tube and network formation in HPMECs >2-fold in a TLR4-dependent manner at 12 h (Fig. 3, A and B). We then performed experiments with conditioned media to determine whether LPS-mediated VEGF-A and Ang2 expression stimulates autocrine angiogenesis. CM from LPS-treated cells or addition of recombinant Ang2 and VEGF-A (both 25 ng/ml) to CM from control cells induced a >3-fold increase in tube and networks (Fig. 3, C and D). The induction of angiogenesis with conditioned media derived from LPS-treated cells was not altered with TLR4 blockade (Ab-TLR4) but was strongly inhibited with a neutralizing antibody against Ang2/VEGF (Ab-Ang2/VEGF) (Fig. 3, C and D). These data demonstrate that LPS stimulates Ang2 and VEGF-A-dependent autocrine angiogenesis in lung microvascular endothelial cells in vitro.

**Nox2 Inhibition Attenuates LPS-mediated Oxidative Stress and Ang2 and VEGF-A Expression in HPMECs**—To determine the source of oxidative stress in LPS-treated cells, Nox2, Nox4, and Nox1 were inhibited in HPMECs using siRNA. Experiments conducted to determine the appropriate dose and duration of silencing revealed that ~60% silencing efficiency for Nox2 and Nox4 was achieved at 48 h and for Nox1 at 36 h (Fig. 4, A and B). We measured superoxide formation in HPMECs after inhibiting Nox2, Nox1, and Nox4. LPS-induced superoxide formation at 15 min was inhibited by >60% in Nox2-silenced cells but did not change significantly in Nox1- and Nox4-silenced cells (Fig. 4C).

To identify the Nox isofom involved in LPS-mediated expression of angiogenic markers in HPMECs, we quantified Ang2, VEGF-A, and Tie2 after inhibiting Nox isoforms. Nox2 silencing, but not Nox1 or Nox4 silencing, suppressed LPS-induced Ang2, VEGF-A, and Tie2 expression at 24 h by >50% (Fig. 4, D and E). These data demonstrate that Nox2 regulates LPS-induced superoxide and Ang2, Tie2, and VEGF-A expression in HPMECs.

**LPS Stimulates Assembly of the Nox2 Complex in HPMECs**—The active Nox2 complex requires both the membrane-bound gp91phox/p22phox subunits along with the cytoplasmic p47phox, p67phox, and RAC2 subunits (14, 16). To demonstrate that all components of the Nox2 complex are present in HPMECs, we quantified the expression of subunits in control

**FIGURE 2.** Systemic LPS induces Tie2 and Ang2 in mouse lung endothelial cells. A, protein from mouse lung endothelial lysates harvested 18 h after intraperitoneal LPS were immunoblotted for Ang2 and Tie2. B, quantification by densitometry is shown for Tie2 and Ang2 from endothelial cells. *, \( p = 0.03 \) (Tie2, control versus LPS); **, \( p = 0.01 \) (Ang2, control versus LPS) (n = 3).
and LPS-treated HPMECs (15 min) by immunoblotting (Fig. 5A). Furthermore, to show that LPS activates Nox2 in HPMECs, we investigated whether p47phox coimmunoprecipitated with gp91phox after LPS treatment. Compared with control cells, we found that p47phox was bound to gp91phox in LPS-treated cells at 15 and 30 min (Fig. 5, B and C). These data suggest that components of the Nox2 complex are present in HPMECs and that LPS treatment results in coimmunoprecipitation of p47phox with gp91phox.

Effect of Nox2 Inhibition on LPS-mediated IKKβ, p38, and JNK Phosphorylation in HPMECs—To investigate the mechanisms underlying Nox-mediated regulation of Ang2, VEGF-A, and Tie2 expression, we examined the effect of LPS on phosphorylation events in the canonical Toll-like receptor signal-
LPS-induced IKKβ/H9252 phosphorylation (Ser177/181) was evident by 5 min, peaked at 10 min, and waned by 30 min (Fig. 6, A and C). Similarly, LPS mediated a temporal increase in p38 (Thr180/Tyr182) and JNK (Thr183/Tyr185) phosphorylation, with a peak at 60 min (Fig. 6, B and D). In Nox2-silenced cells, but not Nox4 or Nox1-silenced cells, LPS-induced IKKβ/H9252 phosphorylation (10 min) and p38 phosphorylation (60 min) were attenuated significantly (Fig. 7, A–C). The effect of Nox isoform silencing on JNK phosphorylation was not selective and showed a marginal reduction. These data show that Nox2 inhibition attenuates IKKβ and p38 phosphorylation in HPMECs.

NF-κB and AP-1 Activation Induced by LPS Is Modulated by Nox2 Silencing in HPMECs—The promoters of Ang2, VEGF-A, and Tie2 have transcription factor binding sites for NF-κB and AP-1 (25–28). To examine the role of Nox isoforms in the activation of transcription factors that regulate expression of angiogenesis genes in HPMECs, we quantified NF-κB and AP-1 in cellular nuclear extracts obtained after LPS treatment. LPS-induced an 8.1- and 3.4-fold increase in NF-κB and AP-1 levels at nuclear extracts, respectively, supporting activation of these transcription factors (Fig. 8, A and B). Nox2 silencing, but not Nox4 or Nox1 silencing, attenuated an LPS-mediated increase in nuclear translocation of these transcription factors.
Nox2 Regulates LPS-mediated Ang2 Signaling

Effect of Nox2 Silencing on LPS-mediated Autocrine Angiogenesis in HPMECs—To determine whether Nox2 regulates LPS-mediated angiogenesis in HPMECs, we used both cell-based and CM experiments. LPS-induced tube and network formation was attenuated by >60% in Nox2-silenced cells (Fig. 9, A and B). Supplementing Nox2-silenced cells with recombinant Ang2 and VEGF-A restored LPS-mediated angiogenic tube and network formation (Fig. 9, A and B). Similarly, CM from LPS plus siNox2-treated cells showed a marked reduction in stimulating HPMEC angiogenic responses compared with CM from LPS-treated cells (Fig. 9, C and D). Supplementation of CM from Nox2-silenced cells with recombinant Ang2, recombinant VEGF-A, or both fully restored LPS-mediated angiogenic tube and network formation (Fig. 9, C and D). These data support the role of Nox2 in regulating LPS-induced, Ang2/VEGF-A-dependent angiogenesis in HPMECs.

DISCUSSION

Recent studies demonstrate the importance of pulmonary endothelial Ang2 expression to lung inflammation and vascular injury in BPD and other diseases (29, 30). However, the mechanisms by which Ang2 is regulated in response to systemic sepsis remain unclear. In this study, we report a novel role for Nox-dependent signaling in the regulation of proinflammatory Ang2 expression in pulmonary endothelial cells. We demonstrate that Nox2 regulates LPS-mediated Ang2 and VEGF-A expression as well as Ang2- and VEGF-A-dependent autocrine angiogenesis in HPMECs. We propose that Nox2 regulates LPS-mediated IKKβ and p38 phosphorylation, resulting in the nuclear translocation of NF-κB and AP-1, transcriptional induction of VEGF-A and Ang2, and altered angiogenesis in HPMECs (Fig. 10). Validating the importance of this mecha-
The mechanisms underlying the regulation of proinflammatory Ang2 expression in the endothelium by reactive oxygen species (ROS) remain unknown. In HPMECs, LPS-induced superoxide formation and Ang2/VEGF-A expression were inhibited by Nox2 silencing. The presence of Nox2 subunits in HPMECs as well as coimmunoprecipitation of p47phox with gp91phox after LPS treatment support activation of the Nox2 complex. Studies with chemical inhibitors that inhibit Nox (VAS2870) or quench superoxide (PEG-superoxide dismutase) suggest that superoxide formation contributes to LPS-mediated induction of angiogenic markers. In conjunction with our Nox2 silencing data, these results support a role for Nox2-induced ROS in LPS-mediated Ang2 and VEGF-A expression. A direct role for Nox in inflammatory Ang2 expression has not been shown before. In a hemangiomma model, Bhandarkar et al. (38) demonstrated that lentiviral knockdown of Nox4 impaired Ang2 expression in polyoma-transformed brain endothelial cells. Although their data support our observation that Nox isoforms can regulate Ang2 expression, their tumor model is likely representative of Ets-driven Ang2 expression, whereas our inflammatory model is more reflective of AP-1/NF-κB-driven Ang2 up-regulation. Although other investigators have shown that VEGF signaling in the endothelium activates Nox, the role of Nox isoforms in regulating Ang2 expression in polyoma-transformed brain endothelial cells is Nox-2-dependent (39). Oh et al. (40) have reported previously that, in microglial cells, Nox indirectly regulates VEGF expression through hypoxia-inducible factor 1 (HIF-1) up-regulation. We did not examine HIF-1α specifically in HPMECs because our model did not involve hypoxic conditions and because we were probing proinflammatory VEGF-A expression. Depending on the nature of the stimulus, endothelial VEGF can be up-regulated by transcription factors belonging to the Ets family, NF-κB, and HIF-1α, among others (26). Although Ang1/Tie2-dependent angiogenesis has been reported to involve generation of ROS, the role of Nox isoforms in LPS-mediated Tie2 expression has not been evaluated before (41). Our data suggest that, similar to proinflammatory VEGF-A and Ang2 induction in endothelial cells, Tie2 expression in response to LPS is also modulated by Nox2 in HPMECs.
ined the IKK/NF-κB and MAPK/AP-1 pathways activated in canonical TLR signaling (24). In HPMECs, phosphorylation of IKKβ and the MAP kinase p38 induced by LPS was attenuated by inhibiting Nox2. Although LPS stimulated JNK phosphorylation, our data regarding Nox-silencing and JNK phosphorylation were not selective and not significant. Prior work from Loukili et al. (42) and Menden et al. (13) has demonstrated that Nox isoforms can regulate proinflammatory IKKβ phosphorylation. Although the involvement of ROS in LPS-mediated MAP kinase activation has been reported in other cell types, the specific isoform of Nox that regulates p38 and JNK phosphorylation in lung endothelial cells remains unknown (43–45). Our

FIGURE 9. Effect of Nox2 inhibition on LPS-induced angiogenesis in HPMECs. A, fluorescent microscope images depicting angiogenic tube formation in control, LPS-treated, LPS + siNox2-treated, and LPS + siNox2 + rhAng2 + rhVEGF-A-treated cells. Images were captured at 5× magnification. B, graphical representation summarizing data from four different experiments in HPMECs (n = 4). *, p < 0.001 (control versus LPS tube and network formation); **, p < 0.001 (LPS versus LPS + siNox2 tube and network formation); ***, p < 0.001 (LPS + siNox2 versus LPS + siNox2 + rhAng2 + rhVEGF-A tube and network formation). C, HPMECs were treated with conditioned media from control (CM-control), LPS-treated (CM-LPS), and LPS + siNox2-treated (CM-LPS + siNox2) cells. All cells were treated with Ab-TLR4 before application of conditioned media. Recombinant VEGF-A and Ang2 were used to restore angiogenic responses in cells treated with conditioned media from LPS + siNox2-treated cells (CM-LPS + siNox2 + rhAng2 + rhVEGF-A). D, graphical representation summarizing data from three different experiments in HPMECs (n = 3). *, p < 0.001 (CM-Control versus CM-LPS tube and network formation); **, p < 0.001 (CM-LPS versus CM-LPS + siNox2 tube and network formation); ***, p < 0.001 (CM-LPS + siNox2 versus CM-LPS + siNox2 + rhAng2 tube and network formation); $, p < 0.001 (CM-LPS + siNox2 versus CM-LPS + siNox2 + rhVEGF-A tube and network formation); $$, p = 0.001 (CM-LPS + siNox2 versus CM-LPS + siNox2 + rhAng2 + rhVEGF-A tube and network formation).
data support a role for Nox2 in mediating LPS-induced p38 phosphorylation in HPMECs. Peng et al. (43) showed that LPS-mediated TNF-α expression in cardiomyocytes was dependent on gp91phox and p38 kinase activation. Similarly, Wu et al. (46) and Patel et al. (45) showed Nox2 and Nox4 mediated LPS-dependent MAPK activation in aortic smooth muscle cells and skeletal muscle microvascular endothelial cells, respectively. Our data regarding HPMECs are consistent with the studies mentioned above and support the regulation of proinflammatory MAPK activation by Nox isoforms. The mechanisms underlying the regulation of IKKβ and p38 phosphorylation by Nox2 in HPMECs were not examined in this study because this was not the focus of our experiments. Whether these effects are mediated through TGF-β-activated kinase 1 (TAK1) or represent direct effects on IKKβ and p38 remains to be elucidated. The involvement of ROS in the activation of transcription factors NF-κB and AP-1 has been shown before (47). We examined the nuclear translocation of NF-κB and AP-1 (as a marker of activation) in HPMECs because Ang2, VEGF-A, and Tie2 can be induced transcriptionally by NF-κB and AP-1 (25, 27, 28). We noted that Nox2 silencing, but not Nox4 or Nox1 silencing, inhibited LPS-mediated superoxide formation and NF-κB/AP-1 nuclear translocation. These data support a role for Nox2-dependent ROS in proinflammatory NF-κB and AP-1 activation in HPMECs.

Angiogenesis involves endothelial cell migration, proliferation, and sprouting of new vessels (48). We used an in vitro assay to determine the relevance of Nox2-mediated Ang2 and VEGF-A expression in LPS-induced angiogenesis (23). In HPMECs, LPS-induced Ang2 and VEGF-A expression stimulated angiogenic tube and network formation in an autocrine manner. Nox2 silencing or conditioned media from Nox2-silenced cells attenuated LPS-mediated angiogenic responses, demonstrating the importance of Nox2 in regulating proinflammatory, Ang2-dependent angiogenesis. Although Nox4, Nox1, and Nox2 have been reported to mediate the angiogenic response to hypoxia and growth factors such as VEGF and fibroblast growth factor, the Nox isoform involved in endothin- and Ang2-mediated signaling has not been characterized before (49, 50). Interestingly, supplementation with Ang2 or VEGF-A restored angiogenic responses in Nox2-silenced cells in equal measure, showing that Ang2 alone is sufficient to mediate angiogenesis during inflammation. Our results are consistent with prior work showing that Ang2-dependent endothelial cell migration and sprouting of new vessels requires the presence of inflammatory cytokines or VEGF (7, 11). Examination of the implications of LPS-mediated pulmonary endothelial Ang2, VEGF-A, and Tie2 expression to vascular remodeling in BPD will have to be pursued in animal models.

In summary, we demonstrate that Nox2 regulates Ang2 and VEGF-A expression in pulmonary endothelial cells through the IKKβ/NF-κB and MAPK/AP-1 pathways. We also show that Ang2 and VEGF-A mediate LPS-induced angiogenic responses in an autocrine fashion. To the best of our knowledge, this is one of the initial reports to demonstrate regulation of Ang2 expression and proinflammatory angiogenesis by Nox2-dependent signaling. Increased Ang2 expression in the systemic circulation or in the lung has been associated with mortality in humans with sepsis, severity of acute lung injury, and with development of BPD in premature infants (30, 31, 51). Although we showed that mouse lung endothelial cells express increased Ang2 and Tie2 after systemic LPS, the rest of our data were obtained in primary cells in vitro, and, therefore, verification of the results of this study in animal models expressing angiogenic factors in a tissue-restricted manner will enable us to better understand the significance of these findings. This assumes translational significance because inhibiting Ang2 using antibodies or modulating Nox2 activity are emerging as promising strategies to decrease lung injury in bacterial sepsis (51, 52).

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FIGURE 10. Proposed mechanism for Nox2-dependent regulation of LPS mediated Angiopoietin signaling and angiogenesis in HPMECs.
Nox2 Regulates LPS-mediated Ang2 Signaling

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