LPS-mediated Angiopoietin-2 dependent autocrine angiogenesis is regulated by Nox2 in human pulmonary microvascular endothelial cells*

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*Running title: Nox2 regulates LPS-mediated Ang2 signaling

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ABSTRACT
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 lipopolysaccharide (LPS) mediate Ang2 signaling in human pulmonary microvascular endothelial cells (HPMEC) 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 IKKβ and p38 phosphorylation, and nuclear translocation of NF-κB and AP-1. In HPMEC, 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 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 HPMEC 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 hallmarks 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 lipopolysaccharide (LPS) released during sepsis induce a change in the endothelial phenotype from a quiescent phenotype to a “pro-inflammatory” 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). Further, Ang2
and Ang1 expression are temporally regulated in the developing lung and facilitates development of 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 auto-phosphorylation (6, 7). Constitutive Ang1/Tie2 signaling promotes endothelial survival, migration, and antagonizes the pro-inflammatory effects of cytokines and VEGF in the mature endothelium (7, 11). Ang2 competes with Ang1 for Tie2 and promotes expression of cellular adhesion molecules such as vascular cell adhesion molecule (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). While systemic LPS administration has been shown to augment Ang2 expression in the liver, lung, and other tissues the mechanisms involved in the 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 pro-inflammatory 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 ischemia-reperfusion, inflammation, and cell death (14, 16). Nox2, Nox4, and Nox1 have all been previously shown to mediate cytokine expression in response to LPS in endothelial cells of varied lineage (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 (HPMEC). Herein, we demonstrate that Nox2 regulates LPS-mediated Ang2 and VEGF-A expression in HPMEC through the IKKβ/NF-κB 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 human pulmonary microvascular endothelial cells (HPMEC) from ScienCell (Carlsbad, CA, USA) were used between passages 3–5 for all experiments. HPMEC 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 lipopolysaccharide (LPS, 100 ng/ml) and human TLR4 neutralizing antibody (Ab-TLR4, 5 µM) were purchased from Invivogen (San Diego, CA, USA). Tiron, potassium phosphate, EGTA, sucrose, lucigenin, and NADPH were all purchased from Sigma (St. Louis, MO, USA). Collagenase, FBS, DMEM, and PEG-SOD were purchased from Sigma (400 U/ml). VAS2870 [3-benzyl-7-(2-benzoxazolyl) thio-1,2,3-triazolo (4,5-d) pyrimidine, 10 µM], a reversible Nox inhibitor, was obtained from Vasopharm (kind gift of Dr. Reinhard Schinzel, Würzburg, Germany). Recombinant VEGF-A (rhVEGF-A, 25 ng/mL) and Ang2 (rhAng2, 25 ng/mL) were purchased from R&D Systems (Minneapolis, MN, USA). A combined neutralizing antibody against Ang2 and VEGF (Ab-Ang2/VEGF, 500 ng/ml) was a kind gift from Roche Innovation Center (Pharma Research and Early Development, Penzberg, Germany).

**Mice:** Care of the mice before and during the experimental procedures was conducted in accordance with the policies of the Biomedical Resource Center, Medical College of Wisconsin, and the National Institutes of Health guidelines for the care and use of laboratory animals. All protocols had received
prior approval from the Medical College of Wisconsin Institutional Animal Care and Use Committee. C57BL/6 mice were obtained from Charles River Laboratories (Franklin, CT).

Isolation of endothelial cells from murine lungs: For endothelial cell isolation, cells from 2-3 neonatal C57BL/6 pups (7 days old) were pooled per condition. Mice were injected intraperitoneally with 1mg/kg LPS or saline, and lungs were harvested after 18 hr following sacrifice of animals. Harvested lungs were minced with sterile scissors in ice-cold DMEM, and then transferred to 15mL of pre-warmed 1mg/mL Collagenase solution in DMEM. The mixture was allowed to rotate for 45 minutes at 37°C. The digested tissue was then passed through a 14g cannula attached to a 20mL syringe several times, followed by passage through a 70 µm cell strainer, and washed with 20% FBS + DMEM. Cells were then centrifuged at 400 x g for 5 minutes and the supernatant was aspirated off. The cell pellet was re-suspended with 0.1% BSA in PBS. The suspension was incubated with anti-PECAM-1 antibody-conjugated Dynabeads™ from Life Technologies, Carlsbad, CA, USA (prepared according to the manufacturer’s protocol) in a rocker for 15 min at room temperature. Upon completion, the cells were washed with PBS 3x and the proteins were extracted with RIPA buffer following standard protocol.

Quantification of Ang2, VEGF-A, Tie2, Nox1, Nox2, and Nox4 mRNA expression using real-time PCR: Total RNA was extracted from HPMEC using the RNeasy Mini Kit from Qiagen (Valencia, CA, USA), and cDNA was synthesized from 1 µg of RNA using the iScript cDNA synthesis kit from Bio-Rad (Hercules, CA, USA), according to the manufacturer’s instructions. The transcripts were amplified and gene expression data was collected on a Bio-Rad IQ5 with SYBR Green Mastermix from Bio-Rad. In experiments using PEG-SOD and VAS2870, the chemicals were incubated with the cells for 1 hr prior to LPS treatment. The primers for Ang2, VEGF-A, and Tie2 were obtained from Operon (Huntsville, AL, USA). They consisted of Ang2 (sense-GAGGAAGCTG-TCTCGAAGCTTG, anti-sense-GGGCAGAATGCACTTCG), VEGF-A (sense-GGGGACGATCATACGAAGGT, anti-sense-ATCTGCATGG-TGATGTGGGA), and Tie2 (sense-TACTAATT-GAAGAAATGACCCCTGG, anti-sense-GGAG-TGTGTAAATGTGGAAATC T). The primers for Nox1, Nox2, and Nox4 were purchased commercially from SCBT (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the primers for 18s were purchased commercially from MCLabs (San Francisco, CA, USA). 18s was used as the housekeeping gene. Relative gene expression of Nox1, Nox2, Nox4, Ang2, VEGF-A, and Tie2 were calculated by the Pfaffl method (18).

Immunoblotting for quantifying changes in protein expression and phosphorylation: Whole cell lysates were prepared from HPMEC by lysing cells with a modified RIPA buffer containing commercially available protease and phosphatase inhibitors (Sigma). Protein quantification was done using a Bicinchoninic Acid (BCA) protein assay from Thermo Fisher (Rockford, IL, USA) according to the manufacturer’s instructions using bovine serum albumin (BSA) as standard. Immunoblotting was done following standard protocol. The primary antibodies for angiogenic expression were: mouse anti-Tie2 (Cell Signaling, Danvers, MA, USA, 1:1000), rabbit anti-Ang2 (Abcam, Cambridge, MA, USA, 1:1000), rabbit anti-VEGF-A (SCBT, 1:500), and mouse anti-β-Actin (Sigma, 1:5,000). For phosphorylation, the primary antibodies were: rabbit anti-(p)IKKβ, rabbit anti-(p)p38 MAPK, rabbit anti-(p)SAPK /JNK, rabbit anti-p38 MAPK, rabbit anti-SAPK /JNK (antibodies purchased from Cell Signaling, 1:1000), and mouse anti-IKKβ (SCBT, 1:500). The antibodies used on Nox2 components were: rabbit anti-Rac2 (SCBT, 1:500), rabbit anti-p22phox (SCBT, 1:500), rabbit anti-p47phox (SCBT, 1:500), mouse anti-p67phox (BD Biosciences, San Jose, CA, USA, 1:500), and goat anti-gp91phox (SCBT, 1:500). Blots were developed using enhanced chemiluminescence (ECL) and blots were stripped for 15 min if needed with Restore Plus stripping buffer (Thermo Fisher). β-Actin or the corresponding non-phosphorylated antibody was used for normalization, and densitometry was performed using ImageJ Software (NIH, Bethesda, MD).

Detection of NADPH-dependent superoxide formation: NADPH-dependent production of superoxide was quantified using a Tiron-
inhibitable lucigenin chemiluminescence assay as done previously (19, 20). Briefly, 1x10^4-2x10^4 cells were seeded in a white 96-well plate (Bio-Rad) and grown for 24 hr. Cells were treated with LPS (100 ng/mL) for 7-45 minutes and each condition run in quintuplicate, the Tiron-inhibition (5 μM) wells run in duplicate, and a blank well run with only lucigenin. After LPS incubation, cells were briefly washed with Hepes Buffered Saline two times, following the last wash 100 μL of 50 μM potassium phosphate buffer with 1 mM EGTA and 1:1000 protease inhibitor cocktail (Sigma) was added to each well. Immediately following 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). Following 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 (RLU) summated for each well at the end of 10 min (luminometer from Turner Scientific, Madison, WI, USA). 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 (RLU).

**siRNA-mediated Nox1, Nox2, and Nox4 gene silencing:** siRNA sequences targeting Nox1, Nox2, and Nox4 were purchased from SCBT, 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 the siRNA transfection medium (SCBT). The plates were then incubated with either the control or siRNA strand (1 μg) in transfection medium and incubated for 16 hours. Subsequently, the reagents were aspirated off, and normal ECM was gently put on the plates. Optimal period of silencing was determined as 36 hours for Nox1 and 48 hours for Nox2 and Nox4 using mRNA and protein studies. The silencing efficiency was determined by PCR (primers mentioned above) and by immuno-blotting using goat anti-Nox1 (1:500), goat anti-Nox2 (1:500), and rabbit anti-Nox4 (1:500) antibodies purchased from SCBT. β-Actin was used for normalization.

**Immunoprecipitation of Nox2:** Cells grown to the 90% confluence in 50-mm dishes were treated with LPS for 15 or 30 min or left untreated. The beads were prepared with the goat anti-gp91phox antibody (SCBT) as per the manufacturer’s protocol using SureBeads™ protein G magnetic beads for immuprecipitation (BioRad). 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 2x Laemmli 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 (SCBT, 1:500) and rabbit anti-p47phox (SCBT, 1:500). Blots were developed using enhanced chemiluminescence (ECL), and densitometry was performed using ImageJ Software (NIH).

**Cellular fractions and transcription factor assays:** Nuclear and cytoplasmic fractions were obtained with the use of a commercially available nuclear extraction kit (Cayman Chemicals, Ann Arbor, MI, USA). The nuclear fractions obtained were quantified using a standard BCA assay (Thermo Fisher) following the manufacturer’s protocol. 5 μg of nuclear extract was used per well with the NF-κB (p65) transcription factor ELISA (Cayman Chemicals) and TRANSAM™ AP-1 c-JUN (Active Motif, Carlsbad, CA, USA) as per the protocols provided by the manufacturer, and all samples were run in duplicate. The nuclear fractions were immunoblotted with rabbit anti-histone (Epitomics, Burlingame, CA, 1:5000) to normalize for protein loading.

**Angiogenic tube and network formation assay in Matrigel:** HPMEC grown to ~80% confluence in 6-well culture plates were either silenced or left unsilenced. After 36 hr of silencing, media was replaced with serum-free media, followed by treatment with LPS for 10 hr. Cells were then detached with Tryple Express (Life Technologies), re-suspended in basal ECM and 6x10^4 cells in 300 μL of media were plated on to a 24-well matrigel matrix coated plate (BD Biosciences). Angiogenesis was assessed 12 hr after cells were seeded on
null regulated LPS-mediated Ang2 signaling  

matrigel. For experiments using the human TLR4 neutralizing antibody (Ab-TLR4, 5 µM) the cells were treated with the inhibitor for 1 hr prior to LPS incubation. For rescue experiments with rhAng2 and rhVEGF-A, cells were treated for 10 hr prior to seeding in matrigel. Calcein AM (BD Biosciences) fluorescent dye was used to enhance the visibility of tube and network formation in Matrigel. Angiogenesis was evaluated by counting the number of tube and network formations in one quadrant (the same one for each condition) and multiplying by four. Only tubular structures connecting 2 cell clusters were considered for measurements, whereas cell clusters with at least 3 tubular structures emanating out were considered to be a network. Representative images were taken using a Zeiss Observer Z1 fluorescence microscope with attached camera at 5x zoom.

For experiments with conditioned media (CM), HPMEC supernatants were collected 24 hr after LPS treatment from control, LPS-treated, and LPS+siNox2-treated cells grown in serum free media. HPMEC grown to 75% confluence in a 6-well culture plate were treated with conditioned media from different conditions for 10 hr. A TLR4 neutralizing antibody (Ab-TLR4, 5 µM) was used to block LPS signaling in all experiments with conditioned media. Cells were then detached with Tryple Express, and the matrigel assay performed as above. For rescue experiments with rhAng2 and rhVEGF-A, cells were treated for 10 hr prior to seeding in Matrigel. For experiments with Ab-Ang2/VEGF (blocking antibody), HPMEC were treated with 500 ng/mL of antibody for 30 min before addition of conditioned media (CM).

Statistical analysis: Statistical analysis was done using STATA 12 (StataCorp LP, Dallas, TX). Data are presented as mean ± SD. P<0.05 was considered significant for experiments. Fold changes in protein levels relative to the untreated, control cells were quantified by densitometry and were compared between various treatments using ANOVA. Changes in superoxide levels, transcription factor levels, and angiogenic formations relative to the untreated, control cells were compared between using ANOVA. The Bonferroni test was used in conjunction with ANOVA 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 ANOVA.

RESULTS  

LPS-induced Ang2, Tie2, and VEGF-A expression in HPMEC 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 HPMEC. LPS-induced Ang2, Tie2, and VEGF-A RNA expression was evident at 3 hr, peaked by 7 hr, and decreased by 24 hr (Fig. 1A). Correspondingly, LPS mediated expression of Ang2, Tie2, and VEGF-A protein was detectable by 12 hr, peaked at 24 hr, and decreased by 36 hr (Fig. 1B, 1C). TLR4 (LPS recognition receptor) blockade using a specific antibody resulted in a dose-dependent inhibition of Ang2 and VEGF-A expression (Fig. 1D & 1E). These data show that LPS mediates TLR4-dependent expression of angiogenic markers in HPMEC.

To assess the effect of LPS on oxidative stress in HPMEC 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 figure 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-SOD (a superoxide anion scavenger) (21, 22). Pre-treatment with VAS2870 (10 µM) or PEG-SOD (400 U/mL) for 1 hr attenuated LPS mediated induction of Ang2, VEGF-A, and Tie2 RNA at 3 hr (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 hr after intraperitoneal LPS (1 mg/kg) injection in 7 day-old mice. Mouse lung endothelial cells showed >2.2-fold increase in Tie2 and Ang2 expression after systemic LPS (Figs. 2A, 2B). These data demonstrate that LPS induces Tie2 protein in lung endothelial cells in neonatal mice.

**LPS stimulates angiogenic tube and network formation in HPMEC 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 HPMEC by >2-fold in a TLR4-dependent manner at 12 hr (Figs. 3A, 3B). We then performed experiments with conditioned media to determine whether LPS-mediated VEGF-A and Ang2 expression stimulates autocrine angiogenesis. Conditioned media (CM) from LPS treated cells or addition of recombinant Ang2 and VEGF-A (both 25 ng/mL) to conditioned media from control cells (3D). 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. 3C, 3D). 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, VEGF-A expression in HPMEC:** To determine the source of oxidative stress in LPS treated cells, Nox2, Nox4, and Nox1 were silenced in HPMEC 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 hr, and for Nox1 at 36 hr (Figs. 4A, 4B). We measured superoxide formation in HPMEC 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 isoform involved in LPS mediated expression of angiogenic markers in HPMEC 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 hr by >50% (Figs. 4D, 4E). These data demonstrate that Nox2 regulates LPS-induced superoxide and Ang2, Tie2, and VEGF-A expression in HPMEC.

**LPS stimulates assembly of the Nox2 complex in HPMEC:** 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 HPMEC we quantified expression of subunits in control and LPS treated HPMEC (15 min) by immunoblotting (Fig. 5A). Further, to show that LPS activates Nox2 in HPMEC we investigated whether p47phox co-immunoprecipitated with gp91phox after LPS treatment. Compared to control cells, we found that p47phox was bound to gp91phox in LPS treated cells at 15 and 30 min (Figs. 5B, 5C). These data suggest that components of Nox2 complex are present in HPMEC and LPS treatment results in co-immunoprecipitation of p47phox with gp91phox.

**Effect of Nox2 inhibition on LPS mediated IKKβ, p38 and JNK phosphorylation in HPMEC:** 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 signaling pathway (24). LPS-induced IKKβ phosphorylation (Ser177/181) was evident by 5 min, peaked at 10 min and waned by 30 min (Figs. 6A, 6C). Similarly, LPS mediated a temporal increase in p38 (Thr180/Tyr182) and JNK (Thr183/Tyr185) phosphorylation with a peak at 60 min (Figs. 6B, 6D). In Nox2-silenced cells, but not Nox4 or Nox1-silenced cells, LPS-induced IKKβ phosphorylation (10 min) and p38 phosphorylation (60 min) were attenuated significantly (Figs. 7A, 7B, 7C). The effect of Nox isoform silencing on JNK phosphorylation was not selective and showed marginal reduction. These data show that Nox2 inhibition attenuates IKKβ and p38 phosphorylation in HPMEC.

**NF-κB and AP-1 activation induced by LPS is modulated by Nox2 silencing in HPMEC:** 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 HPMEC 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 1 hr in nuclear extracts, respectively supporting activation of these transcription factors (Figs. 8A, 8B). Nox2 silencing, but not Nox4 or Nox1 silencing, attenuated LPS mediated increase in nuclear translocation of these transcription factors by >60% (Figs. 8A, 8B, 8C). Nox4 silencing has a minimal (<10%) effect on NF-κB activation alone (Fig. 8A). These data suggest that NF-κB and AP-1 activation in response to LPS in HPMEC is predominantly Nox2-dependent.

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

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 pro-inflammatory 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 HPMEC. 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 HPMEC (Fig. 10). Validating the importance of this mechanism in animal models of BPD will further support the biological relevance of our findings.

The temporal increase in Ang2 and VEGF-A expression observed with LPS in HPMEC is consistent with rodent and human studies showing increased expression of Ang2 or VEGF-A in the whole lung or systemic circulation after LPS administration or systemic sepsis, respectively (12, 31–33). Our data indicate that pulmonary microvascular endothelial cells respond to bacterial ligands with robust Ang2, Tie2, and VEGF-A expression. Previous work on the relationships between Tie2 expression and sepsis has noted disparate results. Our data is consistent with Heijden et al. (34) and Yang et al. (35) who showed that Tie2 expression in the systemic circulation was elevated in sepsis, but are in contrast with results reported by Mofarrahi et al. (12) and David et al. (36) who showed decreased lung Tie2 gene expression after LPS administration. The differences in these results could be explained by the fact that while our data were obtained in HPMEC, the above studies examined Tie2 expression in whole lung or systemic circulation. Our data in mouse lung endothelial cells showing that Tie2 and Ang2 expression is induced after systemic LPS support our in vitro results. Similar to our findings, in a model of tumor necrosis factor-α induced microvascular injury William et al. (37) noted upregulation of Tie2 in human umbilical vein endothelial cells and coronary microvascular endothelial cells. The presence of AP-1 binding sites in the promoter region of the human Tie2 gene further supports our data (28). Increased expression of Ang2, VEGF-A, and Tie2 in parallel with NF-κB and AP-1 activation is consistent with the establishment of a “pro-inflammatory” endo-
Nox2 regulates LPS-mediated Ang2 signaling

The mechanisms underlying regulation of pro-inflammatory Ang2 expression in the endothelium by reactive oxygen species (ROS) remain unknown. In HPMEC, LPS-induced superoxide formation and Ang2/VEGF-A expression were inhibited by Nox2 silencing. The presence of Nox2 subunits in HPMEC as well as co-immunoprecipitation 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-SOD) 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 haemangioma model, Bhandarkar et al. (38) demonstrated that lentiviral knockdown of Nox4 impaired Ang2 expression in polyoma transformed brain endothelial cells. While their data supports our observation that Nox isoforms can regulate Ang2 expression, their tumor model is likely representative of Ets-driven Ang2 expression while our inflammatory model is more reflective of AP-1/NF-κB driven Ang2 upregulation. While other investigators have shown that VEGF signaling in the endothelium activates Nox, we demonstrate that LPS-induced VEGF expression in lung endothelial cells is Nox-2 dependent (39). Oh et al. (40) have previously reported that in microglial cells, Nox indirectly regulates VEGF expression through Hypoxia-inducible factor 1 (HIF-1) upregulation. We did not examine HIF-1α specifically in HPMEC as our model did not involve hypoxic conditions and because we were probing pro-inflammatory VEGF-A expression. Depending on the nature of the stimulus, endothelial VEGF can be upregulated by transcription factors belonging to the Ets family, NF-κB, and HIF-1α among others (26). While 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 pro-inflammatory VEGF-A and Ang2 induction in endothelial cells, Tie2 expression in response to LPS is also modulated by Nox2 in HPMEC.

To examine the mechanisms underlying Nox mediated regulation of Ang2 and VEGF expression in HPMEC we examined the IKKβ/NF-κB and MAPK/AP-1 pathways activated in canonical TLR signaling (24). In HPMEC, phosphorylation of IKKβ and the MAP kinase p38 induced by LPS was attenuated by inhibiting Nox2. Although LPS stimulated JNK phosphorylation, our data with regards to Nox-silencing and JNK phosphorylation were not selective, and were not significant. Prior work from Loukili et al. (42) and Menden et al. (13) has demonstrated that Nox isoforms can regulate pro-inflammatory IKKβ phosphorylation. While 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 the lung endothelial cells remains unknown (43–45). Our data support a role for Nox2 in mediating LPS-induced p38 phosphorylation in HPMEC. 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 cell and skeletal muscle microvascular endothelial cells, respectively. Our data in HPMEC is consistent with the above studies and supports the regulation of pro-inflammatory MAPK activation by Nox isoforms. The mechanisms underlying regulation of IKKβ and p38 phosphorylation by Nox2 in HPMEC were not examined in this study as 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 remain 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 nuclear translocation of NF-κB and AP-1 (as a marker of activation) in HPMEC as Ang2, VEGF-A, and Tie2 can be transcriptionally induced 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 pro-inflammatory NF-κB and AP-1 activation in HPMEC.

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 HPMEC, LPS-induced Ang2 and VEGF-A expression stimulated angiogenic tube and network 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 pro-inflammatory Ang2-dependent angiogenesis. While 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 endotoxin 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). Examining the implications of LPS mediated pulmonary endothelial Ang2, VEGF-A, and Tie2 expression to vascular remodeling in BPD will need 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 pro-inflammatory angiogenesis by Nox-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). While we showed that mouse lung endothelial cells express increased Ang2 and Tie2 after systemic LPS, the rest of our data was obtained in primary cells in-vitro, and as such verifying 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 as inhibiting Ang2 using antibodies or modulating Nox2 activity are emerging as promising strategies to decrease lung injury in bacterial sepsis (51, 52).
Nox2 regulates LPS-mediated Ang2 signaling

References


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FOOTNOTES
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1To whom correspondence should be addressed: Venkatesh Sampath, Department of Pediatrics, Neonatology- Suite 410, Children’s Corporate Center, 999 N. 92nd Street, Wauwatosa, WI 53226, Tel.: (414) 955-5853; Fax: (414) 266-6979; E-mail: vsampath@mcw.edu
2The abbreviations used are: Ang1, Angiopoietin-1; Ang2, Angiopoietin-2; Tie2, Receptor tyrosine kinase of TIE family; BCA, Bicinchonicic Acid; BPD, Bronchopulmonary dysplasia; BSA, Bovine serum albumin; ECL, enhanced chemiluminescence; ECM, Endothelial cell medium; HIF-1, Hypoxia-inducible factor; HPMEC, Human pulmonary microvascular endothelial cells; LPS, Lipopolysaccharide; Nox, NADPH oxidase; RLU, Relative light unit; ROS, Reactive oxygen species; TLR, Toll-like receptor; VCAM-1, Vascular cell adhesion molecule

FIGURE LEGENDS

FIGURE 1. LPS induces the expression of angiogenic markers and superoxide in HPMEC. A) Ang2, VEGF-A, and Tie2 mRNA expression quantified by real-time PCR 3, 7, and 24 hr after LPS treatment. *p<0.02 (Ang2; Control vs. 3, 7, and 24 hr LPS); **p<0.02 (VEGF-A; Control vs. 3, 7, and 24 hr LPS); ***p<0.01 (Tie2; Control vs. 3 and 7 hr LPS). n=4. B) Cell lysates obtained from LPS-treated or control HPMEC at 12, 24, and 36 hr were immunoblotted for Tie2, Ang2, and VEGF-A. C) Densitometry quantification of angiogenic markers. *p=0.007 (12 hr Tie2: Control vs. LPS); #p=0.003 (12 hr Ang2: Control vs. LPS); $p<0.01 (rest of comparisons between Control vs. LPS for respective proteins). n=4. D) Ang2 and VEGF-A protein expression was quantified in cell-lysates by immunoblotting 24 hr 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 vs. LPS, LPS vs. 2.5 µM, and LPS vs. 5µM); $p<0.03 (VEGF; Control vs. LPS, LPS vs. 2.5 µM, and LPS vs. 5µM.). n=3. F) Superoxide formation in HPMEC was quantified at various time points after LPS treatment using a lucigenin-derived chemiluminescence assay. *p=0.02 (control vs. 7 min LPS); **p<0.001 (control vs. 15 min LPS); ***p<0.02 (control vs. 30 min LPS); $p=0.04 (control vs. 45 min LPS). n=3. G) Lucigenin chemiluminescence was quantified at 1-min intervals in control and LPS (15 min) treated HPMEC as before. *p<0.05 (control vs. LPS, for all comparisons). n=3. H) Ang2, VEGF-A, and Tie2 RNA expression quantified by real-time PCR 3 hr after LPS treatment. *p<0.02 (Ang2; Control vs. 3 hr LPS, LPS vs. LPS+VAS2870, and LPS vs. LPS+ PEG-SOD); **p<0.02 (VEGF-A; Control vs. 3 hr LPS, LPS vs. LPS+VAS2870, and LPS vs. LPS+PEG-SOD); ***p<0.01 (Tie2; Control vs. 3 hr LPS, LPS vs. LPS+VAS2870, and LPS vs. LPS+PEG-SOD). n=3. Cells were pre-treated with VAS2870 and PEG-SOD for 1 hr before LPS.

FIGURE 2. Systemic LPS induces Tie2 and Ang2 in mouse lung endothelial cells. A) Protein from mouse lung endothelial lysates harvested 18 hr after intraperitoneal LPS were immunoblotted for Ang2 and Tie2. B) Quantification by densitometry is shown for Tie2 and Ang2 from the endothelial cells. *p=0.03 (Tie2; Control vs. LPS); **p=0.01 (Ang2; Control vs. LPS). n=3.

FIGURE 3. LPS stimulates angiogenic tube and network formation in HPMEC in an autocrine manner. A) Fluorescent microscope images depicting LPS mediated tube and network formation on Matrigel. HPMEC were treated with LPS with or without a neutralizing TLR4 antibody (Ab-TLR4) for 10 hr,
followed by seeding on Matrigel-coated plates. Tube and network formation was assessed after a further 12 hr. A tube is a connection between two junctions and a network is more than two tubes branching from a junction. Images were taken at 5x magnification. B) Graphical representation summarizing data from four different experiments in HPMEC (n=4). *p<0.001 (control vs. LPS tube and network formation); *p=0.001 (LPS vs. Ab-TLR4+LPS tube and network formation). C) HPMEC were treated with conditioned media from control (CM-control) or LPS-treated cells (CM-LPS) in the presence of Ang2/VEGF-A blockade (CM-LPS+Ab-Ang2/VEGF-A), TLR4 blockade (CM-LPS+Ab-TLR4), or recombinant VEGF-A and Ang2 (CM-control+rhAng2+rhVEGF-A) for 10 hr. Cells were then plated on to Matrigel and angiogenic responses assessed after 12 hr as above. D) Graphical representation summarizing data from three different experiments in HPMEC (n=3). *p<0.001 (CM-Control vs. CM-LPS tube and network formation); **p<0.003 (CM-LPS vs. CM-LPS+Ab-Ang2/VEGF tube and network formation); ***p<0.001 (CM-Control vs. CM-Control+rhAng2+rhVEGF-A tube and network formation).

FIGURE 4. Effect of Nox1, Nox2, and Nox4 silencing on LPS-induced superoxide production in HPMEC. A) mRNA expression of Nox1, Nox2, and Nox4 quantified 48 hr after silencing by real-time PCR. 18s was used as a housekeeping gene. Expression is relative to untreated controls. *p=0.003 (Control vs. Nox1); ***p<0.001 (Control vs. Nox2); **p<0.001 (Control vs. Nox4). n=4. B) Nox1, Nox2, and Nox4 protein expression was quantified in cell lysates by immunoblotting after silencing with respective siRNA. C) Superoxide formation was quantified in HPMEC 15 min after LPS treatment under various test conditions using a lucigenin-derived chemiluminescence assay. *p<0.001 (control vs. 15 min LPS); **p<0.005 (LPS vs. siNox2+LPS). n=3. D and E) Tie2, Ang2, and VEGF-A protein expression was examined in cell lysates 24 hr after LPS treatment by immunoblotting (D), and bands were quantified by densitometry (E). *p<0.001 (Control vs. LPS); **p<0.001 (Control vs. LPS); $p<0.001 (Control vs. LPS); **p<0.001 (Control vs. siNox2+LPS); #p=0.008 (Control vs. siNox2+LPS); $$p=0.008 (Control vs. siNox2+LPS). n=4.

FIGURE 5. Subunits of Nox2 complex are present in HPMEC. A) Nox2 subunits were quantified in control and LPS treated (15 min) HPMEC lysates by immunoblotting. n=3. B and C) gp91phox was immunoprecipitated from control and LPS treated (15 and 30 min) HPMEC, followed by immunoblotting for p47phox (B) with densitometry for the blots shown (C). *p=0.009 (Control vs. 15 min LPS); **p<0.01 (Control vs. 30 min LPS). n=3.

FIGURE 6. Temporal changes in LPS-induced IKKβ, p38, and JNK phosphorylation in HPMEC. A & C) IKKβ phosphorylation was assessed in cell lysates after LPS treatment by western blotting (A), and bands were quantified by densitometry. Mean fold-change in IKKβ phosphorylation relative to untreated controls from four experiments is shown (C). *p<0.001 (Control vs. 5 min LPS); **p<0.001 (Control vs. 10 min LPS); ***p<0.001 (Control vs. 15 min LPS); #p=0.003 (Control vs. 30 min LPS). n=4. B and D) Western blot (B) showing changes in p38 and JNK (MAPK) phosphorylation with LPS treatment at 15, 30, 60, and 120 min. Mean fold-change in p38 and JNK phosphorylation relative to controls was quantified by densitometry (D). *p=0.001 (Control vs. 15 min LPS); **p<0.001 (Control vs. 30 min LPS); ***p<0.001 (Control vs. 60 min LPS) $p=0.001 (Control vs. 60 min LPS). n=4.

FIGURE 7. Effect of Nox inhibition on LPS-induced phosphorylation of p38, JNK, and IKKβ in HPMEC. A) Immunoblot showing changes in phosphorylation of p38, JNK, and IKKβ in control, LPS-treated, siNox2+LPS, siNox4+LPS, and siNox1+LPS-treated cells. B) Densitometric quantification of changes in p38 and JNK (MAPK) phosphorylation relative to controls. *p<0.001 (p38; Control vs. LPS); #p<0.01 (JNK; Control vs. LPS); **p<0.01 (JNK; Control vs. LPS); ***p<0.001 (Control vs. 15 min LPS); **p<0.001 (Control vs. 30 min LPS); ***p<0.001 (Control vs. 60 min LPS) $p=0.001 (Control vs. 60 min LPS). n=5. C) Changes in IKKβ phosphorylation relative to untreated controls was quantified using densitometry. *p<0.001 (Control vs. LPS); **p<0.001 (LPS vs. siNox2+LPS). n=5.
FIGURE 8. Effect of Nox1, Nox2, and Nox4 siRNA on LPS-induced nuclear translocation NF-κB (p65) and AP-1 (c-jun) in HPMEC. A and B) NF-κB and AP-1 levels were quantified 60 min after LPS treatment in nuclear fractions obtained from control, LPS-treated, siNox1+LPS, siNox2+LPS, and siNox4+LPS-treated HPMEC. Nuclear levels are expressed relative to control. *p<0.001 (Control vs. LPS); **p<0.001 (LPS vs. siNox2+LPS); ***p<0.001 (LPS vs. siNox4+LPS); $p<0.001 (Control vs. LPS); $$p<0.001 (LPS vs. siNox2+LPS). n=3. C) Histone expression in nuclear fractions was quantified by immunoblotting as loading control for experiments 8A and 8B.

FIGURE 9. Effect of Nox2 inhibition on LPS-induced angiogenesis in HPMEC. A) Fluorescent microscope images depicting angiogenic tube formation in control, LPS-treated, LPS + siNox2-treated, LPS + siNox2+rhAng2+rhVEGF-A treated cells. Images were captured at 5x magnification. B) Graphical representation summarizing data from four different experiments in HPMEC (n=4). *p<0.001 (Control vs. LPS tube and network formation); **p<0.001 (LPS vs. LPS+siNox2 tube and network formation); ***p<0.001 (LPS+siNox2 vs. LPS+siNox2+rhAng2+rhVEGF-A tube and network formation). C) HPMEC were treated with conditioned media from control (CM-control), LPS-treated (CM-LPS), and LPS + siNox2 (CM-LPS+siNox2) treated cells. Cells were all treated with Ab-TLR4 before application of conditioned media. Recombinant VEGF-A and Ang2 was 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 HPMEC (n=3). *p<0.001 (CM-Control vs. CM-LPS tube and network formation); **p<0.001 (CM-LPS vs. CM-LPS+siNox2 tube and network formation); ***p<0.001 (CM-LPS+siNox2 vs. CM-LPS+siNox2+rhAng2 tube and network formation); $p<0.001 (CM-LPS+siNox2 vs. CM-LPS+siNox2+rhVEGF-A tube and network formation); $$p=0.001 (CM-LPS+siNox2 vs. CM-LPS+siNox2+rhAng2+rhVEGF-A tube and network formation).

FIGURE 10. Proposed mechanism for Nox2-dependent regulation of LPS mediated Angiopoietin signaling and angiogenesis in HPMEC.
Figure 1

**1A**

Nox2 regulates LPS-mediated Ang2 signaling

**1B**

Figure 1B

**1C**

**1D**

Figure 1C

**1E**

**1F**

Figure 1E

**1G**

**1H**

Figure 1G
Figure 2

2A

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Tie2
Ang2
β-actin
CD31

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Relative Fold Change

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Downloaded from http://www.jbc.org/ by guest on September 16, 2017
Figure 3
Figure 4

**4A**
Gene Expression After siRNA

**4B**
Western Blot Images for siNox2, siNox4, and siNox1

**4C**
Luciferase assay results for LPS and siRNA

**4D**
Western Blot Images for control, LPS, siNox2, siNox4, and siNox1

**4E**
Relative Fold Change for Tie2, Ang2, VEGF-A in control, LPS, and siRNA conditions

*Nox2 regulates LPS-mediated Ang2 signaling*
Figure 5

5A

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5C

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* p < 0.05
** p < 0.01
Figure 6

6A

+LPS

Control 5min 10min 15min 30min

(p)IKKβ IKKβ

6B

+LPS

Control 15min 30min 60min 120min

(p)p38 p38 (p)JNK JNK

6C

IKKβ Phosphorylation

Control 5min 10min 15min 30min

+LPS

*p ** ***

6D

MAPK Phosphorylation

Control 15min 30min 60min 120min

+p38 (p)JNK

*p ** *** $
Nox2 regulates LPS-mediated Ang2 signaling
Figure 8

8A

8B

8C

Control  LPS  siNox1  siNox2  siNox4

Histone
Nox2 regulates LPS-mediated Ang2 signaling

Figure 9

9A

Control | LPS | LPS + siNox2 | LPS + siNox2 + rhAng2 + rhVEGF-A

9B

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9C

CM- Control | CM- LPS | CM- LPS + siNox2 | CM- LPS + siNox2 + rhAng2 | CM- LPS + siNox2 + rhVEGF-A

9D

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Nox2 regulates LPS-mediated Ang2 signaling
LPS-mediated Angiopoietin-2 dependent autocrine angiogenesis is regulated by Nox2 in human pulmonary microvascular endothelial cells
Heather Menden, Scott Welak, Stephanie Cossette, Ramani Ramchandran and Venkatesh Sampath

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