Sepiapterin improves angiogenesis of pulmonary artery endothelial cells with in utero pulmonary hypertension by recoupling endothelial nitric oxide synthase

Ru-Jeng Teng,1 Jianhai Du,2 Hao Xu,2 Ivane Bakhutashvili,1 Annie Eis,1 Yang Shi,2 Kirkwood A. Pritchard, Jr.,2 and Girija G. Konduri1

1Division of Neonatology, Department of Pediatrics, and 2Division of Pediatric Surgery, Department of Surgery, Medical College of Wisconsin

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Persistent pulmonary hypertension of the newborn (PPHN) is a common cause of respiratory failure in neonates during their postnatal transition. The impaired vasorelaxation of pulmonary arteries in PPHN is associated with endothelial dysfunction and decreased nitric oxide (NO) production (10, 45). Uncoupled endothelial nitric oxide synthase (eNOS) activity contributes to the reduced NO production in PPHN (23, 24). Impaired angiogenesis contributes to a failure of postnatal adaptation in PPHN (15). Recent studies demonstrated that NO plays a critical role in vascular endothelial growth factor-mediated blood vessel formation (2, 35) and that impaired angiogenesis in PPHN can be reversed by providing exogenous NO (15). The potential role of uncoupled eNOS activity in the mechanisms impairing angiogenesis in PPHN remains unknown.

Tetrahydrobiopterin (BH4) is a critical cofactor which facilitates coupled eNOS activity and eNOS dimer formation, necessary for NO production (29). Factors that regulate cellular BH4 levels include the expression and activity of GTP cyclohydrolase 1 (GCH-1), the rate-limiting enzyme for BH4 biosynthesis, and the conversion of BH4 to its oxidized product dihydrobiopterin (BH2). The affinity of eNOS for BH2 is similar to BH4; however, BH2 is inactive in maintaining eNOS catalytic function (6, 44). An increase in BH2-to-BH4 ratio may then result in BH2 displacing BH4 from eNOS to increase uncoupled eNOS activity (44). Alternatively, BH4 may function as an intracellular antioxidant (25) that improves NO bioavailability. Grobe et al. (16) reported that an increase in lung BH2-to-BH4 ratio contributes to eNOS-derived superoxide (O2·−) formation in a model of pulmonary hypertension induced by aortopulmonary shunt. Farrow et al. (11) recently reported that lung BH2-to-BH4 ratios are increased in the ductus arteriosus ligation model of PPHN. Moreover, Marinos et al. (31) reported that BH4 levels regulate the proliferation of rat coronary artery endothelial cells through increasing NO production. Shimizu and colleagues reported that BH4 reduces NO-induced cell death (41) and enhances in vitro angiogenesis in bovine aortic endothelial cells (42).

On the basis of these previous reports, we hypothesize that 1) intracellular BH4 levels and GCH-1 expression are decreased in pulmonary artery endothelial cells (PAEC) isolated from fetal lambs with PPHN (HTFL-PAEC) compared with PAEC from fetal lambs without PPHN (NFL-PAEC); 2) increasing intracellular BH4 levels, pharmacologically by sepiapterin (Sep), can recouple eNOS in HTFL-PAEC; and 3) increasing intracellular BH4 can improve the angiogenesis in HTFL-PAEC. Our findings suggest a therapeutic potential for Sep in managing PPHN.

MATERIALS AND METHODS

All animal protocols were approved by Medical College of Wisconsin Institutional Animal Care and Use Committee and conformed to the current guidelines of the National Institutes of Health for care and use of laboratory animals. Pulmonary hypertension (HTFL) was induced by fetal ductus arteriosus constriction from 128 ± 2 to 136 ± 2 days gestation as previously described (24). Control fetal lambs (NFL) received sham operation without ductal constriction. After 8 days of ductal constriction, the ewe was euthanized and fetal lungs were removed en bloc. Pulmonary arteries (PA) of the fetal lambs
were dissected into lung parenchyma up to third-generation branches. PAEC were isolated from PA with the use of 0.25% collagenase type A and cells were grown in endothelial growth media (24). Identity of the cells was verified by staining for factor VIII antigen (18) and by acetylated-LDL uptake (46). PAEC were used for experiments between third and sixth passages (6 NFL and 8 HTFL). Passage numbers of NFL-PAEC and HTFL-PAEC were same for all the experiments.

Monoclonal anti-eNOS antibodies were obtained from either BIOMOL (clone H32) or Invitrogen (clone 9D10). Monoclonal anti-heat-shock-protein-90 (hsp90) antibody (clone 68) and growth factor-reduced Matrigel were from BD Biosciences (Bedford, MA). Monoclonal anti-GTP-cyclohydrolase-1 (GCH-1) antibody was from Abnova (Taipei, Taiwan). FITC-conjugated rabbit anti-CD31 (platelet endothelial cell adhesion molecule) antibody was from Abbiotec (San Diego, CA). Bromodeoxyuridine (Brdu) cell proliferation assay kit was obtained from Chemicon International (Temecula, CA). The in situ cell death TUNEL-POD kit was from Roche Applied Science (Indianapolis, IN). Sep, diethylenetriamine NONOate (DETA NONOate), total nitrate/nitrite, 2,4-diamino-6-hydroxy-pyrimidine (DAHP) were from Cayman Chemical (Ann Arbor, MI). All other chemicals were from Sigma (St. Louis, MO). CellTiter 96 Aqueous One Solution was from Promega (Madison, WI). Extraction of total RNA was performed with a RNeasy Mini Kit from Qiagen (Valencia, CA).

Measurement of Intracellular Levels of BH4

BH4 and BH2 were assayed by HPLC with an electrochemical detector (ESA Biosciences CouArray system, model 542) as we previously described (8). Cell pellets were immediately lysed by passing through 28-gauge tuberculin syringe in 50 mM phosphate buffer with pH 2.6, containing 0.2 mM DTPA and 1 mM DTE (freshly added). Samples were centrifuged at 12,000 g for 10 min at 4°C, to remove the debris. The supernatants were filtered through a 10-kDa cutoff column (Millipore, Billerica, MA). BH4 and BH2 were analyzed using a Syngeri Polar-RP column eluted with argon saturated 50 mM phosphate buffer (pH 2.6). Multichannel coulometric detection was set between 0 and 600 mV. One channel was set at −250 mV to verify the reversibility of BH4 oxidative peak detection. Calibration curves were made by summation of the peak areas collected at 0 and 150 mV for BH4 and 280 and 365 mV for BH2. Intracellular concentrations were calculated by using known BH4 and BH2 standards. BH4 and BH2 levels were normalized to cell protein concentration. Ascorbic acid (AA, 35 mM), which recycles BH4 back to BH4 (26), N-acetylcytosine (NAC, 500 μM), or combined Cu/Zn-SOD (400 U/ml) and catalase (CAT, 420 U/ml) were added into the media in some experiments to investigate the effect of antioxidants on the intracellular BH4 and BH2 levels.

GCH-1 mRNA Abundance by Quantitative Real-Time RT-PCR

Primers designed against conserved region of GCH-1 of multiple species were used to perform the real-time RT-PCR as described previously (8, 9). The sequences of the primers are Forward 5′-GGC CGT TTA CTC GTC CAT CCT-3′, Reverse 5′-GGT CTC GTG GTA TCC CTT GGT GAA-3′. Total RNA was extracted by using a RNeasy Mini Kit. Complementary DNA was synthesized from the extracted RNA by use of the iScript cDNA synthesis kit (Bio-Rad). Real-time RT-PCR was performed by iQ5 multicolor real-time PCR detection system (Bio-Rad). The PCR cycle started at 95.0°C for 3 min, then 40 cycles of 95.0°C for 10 s followed by 58.0°C for 1 min and 72°C for 30 s. Number of the threshold cycle (Ct) for each target mRNA was normalized to the corresponding Ct of β-actin mRNA to obtain the ΔCt and then 2^−ΔΔCt was calculated for the corresponding NFL-PAEC to obtain the relative mRNA abundance (30). Immuno precipitation and Immunoblotting

HTFL-PAEC were grown to ~60% confluence, and Sep (30 μM) or isoxanthopterin (IXP, 30 μM) was added to the culture media for 4-h incubation. The concentration of Sep used in this study was based on previous reports showing increased proliferation and/or decreased cell death of endothelial cells (31, 41, 42) at this concentration. Cells were stimulated with ATP (10−5 M) for 10 min after treatment. After being washed with ice-cold HBSS (2X), the plates were placed on liquid nitrogen (3X). The frozen cells were scraped into RIPA buffer containing protease and phosphatase inhibitor cocktails (Sigma). Lysates were preclarified with protein-A Sepharose slurry for 1 h at 4°C before centrifugation at 2,000 rpm for 2 min. The supernatants were incubated with monoclonal anti-eNOS (H32, BIOMOL) overnight at 4°C. Next day, 70 μl of protein-A Sepharose slurry was added and mixed for 1 h at 4°C. After centrifuging at 2,000 rpm for 5 min and removal of the supernatant, 70 μl of 2X Laemmli buffer was added to the beads, and the mixture was heated at 95°C for 10 min, to elute precipitated proteins. The immunoprecipitated proteins were separated by 7.5% SDS-PAGE and transferred to a nitrocellulose membrane. Monoclonal anti-eNOS antibody (9D10, 1:500) and monoclonal anti-hsp90 antibody (1:1,000) were used to identify protein signals on the membrane. Cell lysates of NFL-PAEC and HTFL-PAEC were also separated by 15% SDS-PAGE and blotted with monoclonal anti-GCH-1 antibody (1:500) followed by horseradish peroxidase-conjugated anti-mouse IgG antibody (Bio-Rad, 1:9,000). Signals were generated by using SuperSignal West Pico (Pierce) and recorded on CL-Xposure films (Pierce). The integrated optical density (IOD) was processed by Image J. Hsp90 IODs were divided by the corresponding IODs of eNOS and PAEC treated with ATP as used the standard for each experiment. Low-temperature electrophoresis was used to investigate the eNOS dimer formation (22).

In Vitro Angiogenic Activities

Cell growth, apoptosis, proliferation, tube formation, monolayer scratch recovery, and invasion assays were used to evaluate the angiogenic activities of PAEC by using previously published protocols from our laboratories (43) and those of Gien et al. (15).

Cell Growth and Proliferation

Cell growth was assessed by intracellular reduction of [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTT or Owen’s reagent) into formazan (5) red-free RPMI 1640 with 20% FCS were plated in each well of a 96-well plate with or without Sep (30 μM) and incubated for 48 h at 37°C, 5% CO2 and 95% humidified room air. The assay solution, 20 μl per well, was added and the 96-well plate was returned to 37°C for a second 3 h. Absorbance at 490 nm was used to estimate cell numbers.

Brdu incorporation was used to estimate PAEC proliferation. PAEC (2×104) were seeded in each well of a 96-well plate and were incubated in DMEM containing 20% FCS at 37°C and at 5% CO2 with 95% humidified room air. Cells were allowed to attach and then serum starved for overnight incubation. The medium was again changed to DMEM supplemented with 20% FCS for assessing appropriate proliferation and Brdu, with or without Sep (30 μM), for 24 h in 37°C at 5% CO2. The plate was washed and fixed before addition of anti-Brdu antibody and conjugated secondary antibody. Finally, 3′,5′-Tetramethylbenzidine peroxidase substrate was added and absorbance at 450 nm was measured. Proliferation was assessed as being proportional to the increase in absorbance.

Apoptosis

Apoptosis was evaluated by in situ TdT-mediated dUTP nick end labeling (TUNEL) staining as reported by Sgonc et al. (39). PAEC (3

AJP-Lung Cell Mol Physiol • VOL 301 • SEPTEMBER 2011 • www.ajplung.org
× 10^5) in DMEM with 20% FCS were cultured on the Lab-Tek II four-chamber slides for 24 h with or without Sep (30 μM). The cells were fixed with 4% formalin in PBS followed by 3% H2O2 in methanol for 10 min at 25°C to quench the internal peroxidase activity. The cells were then permeabilized with 0.1% Triton X-100 on ice and treated with labeling mixture for 60 min at 37°C in the dark. Next the cells were treated with peroxidase converter solution for 30 min at 37°C and then stained with 3,3′-diaminobenzidine. Eight pictures were taken randomly for each group. The dark brownish-stained nuclei were counted to obtain the percent of apoptosis.

**Tube Formation Assay**

Growth factor-reduced Matrigel (50 μL) was added to each well of a 96-well plate and was allowed to polymerize at 37°C for at least 30 min before cells were added. PAEC (2 × 10^5) in 100 μL of DMEM containing 5% FCS were plated per each well with or without Sep (30 μM). We used lower concentration of serum for these studies to optimize tube formation in the Matrigel system, as described previously (15, 43). IXP (30 μM), a pterin that cannot be converted to BH4, was used as a negative control for Sep (48), DETA-NONOate (20 μM) and Cu,Zn-SOD (400 U/ml) with or without CAT (420 U/ml) were added to the medium, immediately after PAEC were put into the Matrigel system, to study the role of NO and O2⁻ in tube formation. DAHP (5 μM), a GCH-1 inhibitor, was used to study the role of intracellular BH4 in NFL-PAEC tube formation. TSB2, a peptide that inhibits the hsp90 domain that binds eNOS (49), was used to study the role of eNOS-hsp90 association in tube formation. Tubular structures between cell clusters were measured and cell clusters that sent out more than three tubular structures were counted as branching points. Pictures of tube formation were taken at 8 and 14 h after plating at ×200 magnification. Only one picture with best tube formation per well was taken by one of the coauthors who was blinded to the treatment. Total tube length per high-power field was measured for each condition for analyses.

**Monolayer Scratch Recovery Assay**

PAEC were grown to confluence in six-well plates. The cells were serum starved for 30 min in DMEM with 0.5% FCS. Scratch lines were created by 1-ml pipette-tip and the scraped cells were gently rinsed away with HBSS. The medium was then changed back to DMEM with 20% FCS, with or without Sep (30 μM), for 24 h. The narrowest distance of the gap between the frontlines of recovery was measured.

**Cell Invasion Assay**

Matrigel-coated Transwell with 8-μm pores (Chemicon International) was used and 300 μL of serum free DMEM was placed into each insert and incubated at 37°C for 2 h. The serum-free DMEM was then replaced with PAEC (3 × 10^5) suspension in 300 μL. The outer chamber was filled with 500 μL of DMEM, with or without Sep 30 μM, and 5% FCS as the chemoattractant. The plate was incubated at 37°C for 24 h. The inner surface of the insert was thoroughly swabbed with a Q-tip before staining with the staining solution provided with the kit. PAEC that invaded through the 8-μm pores were counted under microscope. Pictures of four randomly selected areas per insert were taken for analysis.

**Ex Vivo Sprouting Angiogenesis**

Growth factor reduced Matrigel was prepared as described for the in vitro tube formation assay. Segments of resistance pulmonary artery, ~0.5 mm long, were placed on top of the Matrigel. DMEM with 20% FCS, with or without Sep 30 μM, was then added and changed every day. The distal-most tip of the tubelike structures was measured after 6 days for comparison. To prove that tubes were endothelial in origin, the artery segments were fixed in 4% buffered formalin and epitopes were exposed by permeabilizing solution (20 mM glycine, 0.005% saponin, in 10 mM PBS). After blocking with 1% FBS in permeabilizing solution, anti-CD31 antibody (1:200) was added for 3 h followed by FITC-conjugated anti-rabbit IgG (1:80) as the secondary antibody for 30 min. Images were taken under Nikon Eclipse TE2000 fluorescence microscope with excitation and emission at 475 and 540 nm, respectively (×20 objective).

**NO2⁻/NO3⁻ Production by PAEC**

Two methods were used to quantify PAEC NO2⁻/NO3⁻ production. PAEC (2 × 10^5) were plated into each well of the 12-well plate, with or without Sep (30 μM), and incubated at 37°C, 5% CO2 and room air for 28 h, and the medium was collected to quantify total NO2⁻/NO3⁻ by Greiss reagent after treatment with nitrate reductase. A similar plate without PAEC was incubated in parallel, with or without Sep (30 μM), as a blank. Total NO2⁻/NO3⁻ concentrations were obtained after subtraction of blank values. Data were normalized to cell protein.

In some studies the culture medium was changed to HBSS with L-arginine (25 μM) and incubated at 37°C, 5% CO2 and humidified 95% room air. ATP (10⁻⁵ M) was then added and incubated for 15 min at same condition. The HBSS was finally collected and analyzed by Sievers Nitric Oxide Analyzer NOA 280i (GE Analytical Instruments, Boulder, CO). HBSS with L-arginine (25 μM) and without cells was used as the blank. Data for stimulated NO2⁻/NO3⁻ production were normalized to cell protein.

**Measurements of O2⁻ Production by DHE Epifluorescence**

Superoxide anion production was evaluated by dihydroethidium (DHE) epifluorescence as we described previously (36). PAEC (10^5) in DMEM supplemented with 5% FCS were seeded into each well of a Lab-Tek II four-well chamber slide and were grown at 37°C to near confluence. The PAEC were then incubated with HBSS containing DHE (10 μM) for 15 min at 37°C to detect the intracellular O2⁻ levels. Fluorescence was imaged by using a Nikon Eclipse TE200 fluorescence microscope with excitation and emission at 510 and 590 nm, respectively. Fluorescence was quantified by using MetaView software and expressed as relative light units.

**Statistical Analyses**

Data were presented as means ± SE. Data were analyzed by t-test to compare two groups if normally distributed. Mann-Whitney U-test was used to compare two groups if data failed the normality test. ANOVA with Student-Newman-Keuls post hoc analysis was used to compare data from more than two groups. A P value < 0.05 was considered as significant. All statistic analyses were done by MedCalc (MedCalc Software, Mariakerke, Belgium).

**RESULTS**

**Intracellular Levels of BH4 in PAEC**

BH4 and BH2 levels were quantified by HPLC. BH2 levels were below the limits of detection but BH4 levels in NFL-PAEC were about 3.5-fold higher than HTFL-PAEC (Fig. 1A, n = 8–12, P < 0.01). Overnight treatment with Sep (30 μM) increased the BH4 levels in HTFL-PAEC by ~70-fold (Fig. 1B, n = 4, P < 0.01), which implied an intact salvage pathway in HTFL-PAEC. Sep is known to inhibit GCH-1 activity with an IC50 of 25 μM (40), so it is unlikely that Sep increases BH4 levels through increased GCH-1 activity. AA increased BH4 levels in HTFL-PAEC by ~73% (Fig. 1C, n = 4, P < 0.001), which may be explained by recycling BH3⁺ back to BH4 (26). The combination of Cu,Zn-SOD (400 U/ml) and CAT (420 U/ml) increased HTFL-PAEC BH4 levels from 38.6 ± 38.6 pmol/mg-protein to 328.1 ± 121.3 pmol/mg-protein (P =
However, BH4 levels did not change when HTFL-PAEC were incubated with NAC (500 μM). These results together suggest that the magnitude of increase in BH4 levels by Sep results from more than just the antioxidant effect.

**mRNA Abundance and Protein Expression of GCH-1 in PAEC**

To explore why BH4 levels are decreased in HTFL-PAEC, GCH-1 mRNA abundance and protein expression were studied. GCH-1 mRNA levels in HTFL-PAEC were 8.0 ± 2.6% (n = 6) of NFL-PAEC by real-time RT-PCR (Fig. 2A). In addition, HTFL-PAEC had lower GCH-1 expression (30.3 ± 8.7%, n = 6) than NFL-PAEC by immunoblotting (Fig. 2B). These findings suggest that the decrease in GCH-1 expression in PPHN is at the transcriptional level.

**In Vitro Angiogenesis Studies**

**Cell growth and proliferation.** Previously we reported that HTFL-PAEC proliferated at a slower rate compared with NFL-PAEC (43). Here we observed increased viable HTFL-PAEC by MTT assay when culture medium was supplemented with Sep (Fig. 3A, n = 8, P < 0.001); however, there was no increase in BrdU incorporation with Sep for both NFL-PAEC and HTFL-PAEC (Fig. 3B, n = 8). BrdU incorporation was lower in HTFL-PAEC, with or without Sep, compared with NFL-PAEC. Our results indicate that the increase in cell counts of HTFL-PAEC by Sep is not due to increased proliferation but due to decreased cell death, as shown below.

**Apoptosis by TUNEL.** We previously showed that apoptosis is increased in HTFL-PAEC and in this study we explored whether Sep decreases apoptosis in HTFL-PAEC by in situ TUNEL. Rates of NFL-PAEC apoptosis were low and unaltered when PAEC cultures were incubated with Sep (Fig. 4, A and B). However, rates of HTFL-PAEC apoptosis were increased compared with NFL-PAEC (Fig. 4C); Sep treatment markedly reduced the percentage of apoptosis in HTFL-PAEC (Fig. 4D, n = 6, P < 0.001). Sep reduced HTFL-PAEC apoptosis to levels similar to NFL-PAEC (Fig. 4E). Together with the findings from the BrdU incorporation assay, the improved cell counts in HTFL-PAEC by Sep may be attributed, in part, to decreased apoptosis.
In vitro tube formation. The total tube length was decreased in HTFL-PAEC compared with NFL-PAEC (Fig. 5, A vs. B, \( n = 6, P < 0.05 \)) as we previously reported (43). The presence of Cu,Zn-SOD with CAT or DETA-NONOate added immediately after PAEC were plated increased the tube lengths in HTFL-PAEC (Fig. 5E, \( n = 6, P < 0.05 \)). The results suggest that either removal of reactive oxygen species or an increase in NO availability in the cultures can improve angiogenesis in HTFL-PAEC. To determine whether increasing intracellular BH4 could further increase tube formation in NFL-PAEC, Sep was added into the culture media. Sep did not change the tube lengths in NFL-PAEC. We used DAHP to study the effect of decreasing intracellular BH4, by inhibiting GCH-1 activity, in tube formation. DAHP decreased the NFL-PAEC tube lengths by ~50% (Fig. 5F, \( n = 6, P < 0.05 \)), whereas the addition of Sep recovered the tube lengths in DAHP-treated NFL, which suggests that de novo pathway is critical in BH4 biosynthesis in PAEC and tube formation. To further investigate the role of BH4 in tube formation we treated HTFL-PAEC with Sep. Compared with HTFL-PAEC, the presence of Sep increased the tube lengths in HTFL-PAEC to 79.5 ± 10.7% of NFL-PAEC by 8 h (Fig. 5C). To study whether the effect of Sep on tube formation was mediated by improved eNOS function, we inhibited eNOS function by \( \text{N}^\text{G}-\text{nitro-L-arginine methyl ester} \) (L-NAME). Addition of L-NAME (300 \( \mu \text{M} \)) to the media abolished the beneficial effects of Sep in the HTFL-PAEC (Fig. 5F, \( n = 6, P < 0.05 \)). IXP, a pterin that is not metabolized to BH4, did not change HTFL-PAEC tube lengths (Fig. 5D, \( n = 6, P = 0.35 \)). Sep increased HTFL-PAEC tube lengths by 2.2-fold and 2.7-fold at 8 and 14 h, respectively (Fig. 5F, \( n = 6, P < 0.05 \)). Similar results were observed for branch point (data not shown). Compared with NFL-PAEC without treatment, TSB2, a peptide that induces uncoupled eNOS activity via disrupting eNOS-hsp90 interaction (49), decreased total tube length by ~40% and the addition of Sep abolished the inhibitory effect of TSB2 (Fig. 5G, \( n = 6, P < 0.05 \)). These results suggest that Sep improves tube formation, in part, via recoupling eNOS activity.

Monolayer scratch recovery assay. The monolayer scratch recovery assay examines the ability of PAEC to proliferate and migrate. Similar to our previous report (43), the gaps were significantly wider in HTFL-PAEC (Fig. 6A) than NFL-PAEC (Fig. 6B, \( n = 12, P < 0.01 \)). Gaps in HTFL-PAEC cultures almost disappeared when the cells were treated with Sep (Fig. 6C), whereas Sep did not confer any additional benefit to NFL-PAEC (Fig. 6D). HTFL-PAEC without Sep had the widest gap among the four groups (Fig. 6E, \( n = 12, P < 0.001 \)).

Cell migration and invasion assay. Endothelial cells migrate and invade through the surrounding soft tissue is an initial step
for the sprouting angiogenesis. The number of cells that migrated or invaded through the Matrigel-coated membrane for NFL-PAEC (Fig. 7A) was significantly higher than for HTFL-PAEC (Fig. 7B, n = 6, P < 0.05). Sep did not improve this activity in NFL-PAEC (Fig. 7C), whereas Sep increased the level of cell migration in HTFL-PAEC (Fig. 7D, n = 6, P = 0.02) to that observed in NFL-PAEC cultures (Fig. 7E).

**Ex vivo sprouting angiogenesis.** Sprouting angiogenesis has been used to study the endothelial cell activity in the presence of other cell types. We used the ex vivo sprouting angiogenesis as another assay to study the effect of Sep on HTFL. The distances of the ex vivo tubular structure without Sep treatment in NFL-PA (Fig. 8A) were similar to NFL-PA with Sep treatment (Fig. 8, B and C). The HTFL-PA sprouting angiogenesis (Fig. 8D) increased with Sep (Fig. 8, E and F, n = 6, P < 0.001). The finding in HTFL-PA sprouting angiogenesis was similar to the in vitro angiogenesis assays described above and supports the beneficial effect of Sep on HTFL angiogenesis. The tubular structures (Fig. 8G) were positive for CD31 (Fig. 8 h), confirming their endothelial origin.

**NO$_2^-$/NO$_3^-$ Levels**

Sep significantly increased basal NO$_2^-$/NO$_3^-$ (accumulated over 28 h) in HTFL-PAEC (Fig. 9A, n = 6, P = 0.03). The unstimulated NO$_2^-$/NO$_3^-$ levels measured over 15 min in HBSS buffer (without ATP) were similar in the HTFL-PAEC with or without Sep treatment (4.3 ± 2.0 vs. 5.0 ± 4.0 pmol/mg...
protein). After ATP stimulation for 15 min, NO$_2$/NO$_3$ levels were significantly increased in the Sep-treated NFL-PAEC and HTFL-PAEC (Fig. 9B, $n = 3$, $P = 0.002$), compared with HTFL-PAEC without Sep. The lack of difference in unstimulated NO$_2$/NO$_3$ levels between Sep-treated and control HTFL-PAEC may be a result of low basal production over the 15-min incubation period. The levels measured under basal conditions over the 15-min incubation period are close to the detection limits of the NO analyzer (4). Interestingly, Sep did not increase ATP-stimulated NO$_2$/NO$_3$ production by NFL-PAEC.

**O$_2$ Levels by DHE Epifluorescence**

Baseline DHE epifluorescence in HTFL-PAEC (Fig. 10A) was decreased by Sep (Fig. 10C, $n = 10$, $P < 0.05$). ATP stimulation of HTFL-PAEC increased DHE fluorescence but did not reach statistical significance (Fig. 10B). ATP stimulation did not increase DHE epifluorescence in Sep-treated HTFL-PAEC (Fig. 10D). Sep decreased (~90%) the O$_2$ production by HTFL-PAEC regardless of the presence of ATP (Fig. 10, D and E, $n = 10$). The DHE epifluorescence for NFL-PAEC (Fig. 10, F and G) was lower than HTFL-PAEC and decreased further by Sep both in the absence of ATP (Fig. 10H) and with ATP (Fig. 10I, $n = 10$, $P < 0.05$). ATP stimulation increased the DHE epifluorescence modestly (~70%) in NFL-PAEC whereas Sep decreased the DHE epifluorescence by ~30% (Fig. 10I, $n = 10$, $P < 0.05$) regardless of the presence of ATP stimulation.

**eNOS Dimerization and eNOS-hsp90 Association**

Both eNOS dimer formation and eNOS-hsp90 association are important for eNOS function. We used these two indexes to evaluate Sep effect on eNOS recoupling. The NFL-PAEC eNOS dimer-to-monomer ratios (2.4 ± 0.2) were higher than (Fig. 11A, $n = 5$, $P = 0.0003$) HTFL-PAEC (1.3 ± 0.1). The HTFL-PAEC eNOS dimer-to-monomer ratios (0.33 ± 0.29) increased (5.27 ± 0.75) after Sep treatment (Fig. 11B, $n = 3$, $P = 0.004$). Similar increases in the dimer-to-monomer ratios...
were also observed in NFL-PAEC cultures. The amount of hsp90-eNOS association (as a ratio of hsp90/eNOS) was increased by 7.3 ± 2.3 fold (Fig. 11C, n = 3, P = 0.029) after Sep treatment compared with nontreated group after stimulation of ATP in HTFL-PAEC. No difference in the eNOS-hsp90 association was seen in NFL-PAEC after Sep treatment (Fig. 11D, n = 4, P = 0.204).

**DISCUSSION**

Our studies show that in a lamb model PPHN is associated with a decrease in BH4 levels and decreased expression of GCH-1 enzyme, a rate-limiting step in BH4 synthesis. We also observed that sepiapterin increases BH4 levels and restores eNOS dimerization and coupled eNOS activity in PAEC iso-

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**Fig. 8.** Sep has no effect on ex vivo sprouting angiogenesis of pulmonary arteries (PA) from NFL but increases the ex vivo sprouting angiogenesis of pulmonary arteries from HTFL. NFL-PA without Sep (A), NFL-PA with Sep (B), comparison of sprouting angiogenesis between NFL-PA with and without Sep (C), HTFL-PA without Sep (D), HTFL-PA with Sep (E), comparison of sprouting angiogenesis between HTFL-PA with and without Sep (F), light microscopy of sprouting angiogenesis (G), and positive staining of the tubular structure for CD31 indicates its endothelial origin (H). *P < 0.05 between HTFL-PA with and without Sep.

**Fig. 9.** Sep increases accumulated basal NO2/NO3 production and ATP-stimulated NO2/NO3 production by HTFL-PAEC. The accumulated basal NO2/NO3 production by HTFL-PAEC over 28 h was significantly increased in the presence of Sep, but there was no change in accumulated basal NO2/NO3 production after Sep treatment in NFL-PAEC (A). Sep increases ATP-stimulated NO2/NO3 production, over 15 min, in HTFL-PAEC but not the unstimulated NO2/NO3 production (B). Sep does increase the unstimulated NO2/NO3 production in NFL-PAEC but not the ATP-stimulated NO2/NO3 production (B). *P < 0.05 compared with HTFL-PAEC without Sep; †P < 0.05 compared with the other 3 treatments in HTFL-PAEC.
lated from PPHN lambs. The increase in BH4 levels and coupled activity of eNOS with sepiapterin supplementation is associated with improved angiogenesis and decreased apoptosis in PAEC from PPHN lambs. These findings suggest a phenotypical change of PAEC in PPHN with impaired de novo BH4 formation by altered expression of GCH-1 and downstream effects on eNOS function (32). The alteration in BH4 biosynthesis may underlie the endothelial dysfunction observed in PPHN.

Impaired vasorelaxation and reduced blood vessel density are two characteristic findings in PPHN (14). Importantly, impaired NO production is believed to contribute to both.

Fig. 10. Sep decreases $O_2^\cdot$ production by HTFL-PAEC. Dihydroethidium (DHE) epifluorescence in unstimulated HTFL-PAEC (A), in ATP stimulated HTFL-PAEC (B), in Sep-treated unstimulated HTFL-PAEC (C), and in Sep-treated ATP stimulated HTFL-PAEC (D). Sep decreases the DHE epifluorescence in HTFL-PAEC both with and without ATP stimulation (E). DHE epifluorescence in unstimulated NFL-PAEC (F), in ATP stimulated NFL-PAEC (G), in Sep treated unstimulated NFL-PAEC (H), and in Sep treated ATP stimulated NFL-PAEC (I). Sep also decreases DHE epifluorescence in NFL-PAEC with Sep (J). HSA, HTFL-PAEC with Sep and ATP stimulation; NA, NFL-PAEC with ATP stimulation; NSA, NFL-PAEC with Sep and ATP stimulation; RLU, relative light units. *$P < 0.05$ compared with H and HA. †$P < 0.05$ compared with NA. #$P < 0.05$ compared with N. ¶$P < 0.05$ compared with NS and NSA.
Recent studies demonstrated that eNOS uncoupling (23, 24) and enhanced NADPH oxidase activity (11, 43) contribute to reduced NO bioavailability and impaired angiogenesis in an animal model of PPHN similar to what we used in this study. Our in vitro tube formation assays showed an increase in angiogenesis in HTFL-PAEC when we used a combination of Cu,Zn-SOD and CAT, or an NO donor. These observations suggest that oxidative stress from both increased $O_2^-$ and hydrogen peroxide and depletion of NO impair angiogenesis in PAEC in PPHN.

Coupled catalytic eNOS activity depends on a number of posttranslational modifications including dissociation from caveolin-1 (13, 21), formation of homodimer (1), phosphorylation (21, 29), association with hsp90 (36, 49), and availability of requisite eNOS substrates and cofactors, including L-arginine and BH4 (12). Conditions that affect any of these steps or factors have the potential to uncouple eNOS activity to induce endothelial dysfunction. Previously, we demonstrated that HTFL-PAEC cultures have decreased eNOS-hsp90 association that leads to uncoupled eNOS activity (24). In the present study we observed that HTFL-PAEC have lower intracellular BH4 content, decreased GCH-1 expression, and lower eNOS dimer-to-monomer ratios than NFL-PAEC. Our observations suggest that uncoupled eNOS activity in HTFL-PAEC is mediated by more than one mechanism.

Using crystallography, both Crane et al. (7) and Raman et al. (37) demonstrated the importance of BH4 in stabilizing the eNOS homodimers. Ozaki et al. (33) reported that transgenic overexpression of eNOS in apolipoprotein E knockout mice paradoxically increases vascular $O_2^-$ production because of the uncoupled eNOS activity. However, eNOS/GCH-1 double transgenic mice showed a dramatic increase in both BH4 and NO production, which was not observed in either eNOS or GCH-1 transgenic mice (3). These findings underscore the importance of maintaining an optimal stoichiometric relationship between BH4 and eNOS. Without sufficient BH4, eNOS will not be able to maintain NO production and availability. Our finding of the low BH4 levels in HTFL-PAEC has not been reported before, but similar findings in whole lung tissue were reported by Farrow et al. (11) and can explain our previous finding of the decreased NO levels in HTFL-PAEC (23). In Farrow et al.’s report, there was no difference in BH2 levels between control and hypertensive lambs (11). Different from the reports by both Grobe et al. (16) and Farrow et al., we were unable to show an increased BH2-to-BH4 ratio in HTFL-PAEC, due to undetectable levels of BH2 as previously reported by Huang et al. (19). The observed difference may be attributed to our use of PAEC instead of peripheral lung tissue used in the other studies.

There are several potential mechanisms for the low intracellular BH4 level in HTFL-PAEC, such as reduced GCH-1 expression, impaired GCH-1 function, degradation of GCH-1 by the proteasomes (50), or increased oxidation of BH4 resulting from increased peroxynitrite formation (26, 27). Since the activity of GCH-1 is rate limiting for the generation of BH4 via the de novo pathway (32), our finding that HTFL-PAEC possess lower GCH-1 protein and mRNA levels compared with NFL-PAEC suggests that the low intracellular BH4 concentrations in HTFL-PAEC are due at least in part to downregulation of GCH-1 expression. Although BH4 has been shown to correct endothelial dysfunction under a variety of conditions (34, 47), endothelial cells do not take up BH4 efficiently from the environment. BH4 is oxidized to BH2 before entering the cells (17), whereas Sep, a precursor for BH4, efficiently enters endothelial cells and is rapidly converted to BH4 through the salvage pathway (38) to replenish intracellular BH4 levels. The dramatic increase in BH4 levels in HTFL-PAEC after Sep...
treatment indicates that the salvage pathway in HTFL-PAEC is present and functional.

Recently we reported that impaired angiogenesis in PAEC isolated from PPHN fetal lambs improved after the addition of antioxidants to the tissue culture media (43). Here we demonstrate that Sep is also capable of improving both the in vitro angiogenesis of HTFL-PAEC and pulmonary artery ex vivo sprouting angiogenesis. IXP, which is not metabolized to BH4, was used as a negative control for Sep and did not increase tube formation. TSB2, a peptide that inhibits hsp90-eNOS interactions, also decreased tube formation. Sep not only increased BH4 levels but also increased hsp90-eNOS interaction and restored tube formation. Thus we believe that Sep increases HTFL-PAEC tube formation by an increase in hsp90-eNOS association rather than the antioxidant effect of BH4. Interestingly, Sep did not affect angiogenesis in NFL-PAEC, which indicates that BH4 content is sufficient for NFL-PAEC angiogenesis. However, DAHP inhibition of GCH-1 did impair tube formation in NFL-PAEC, suggesting that intracellular BH4 plays an important role in angiogenesis. One possible mechanism by which Sep improves angiogenesis in HTFL-PAEC is through an increase in NO production (35). The increase in NO levels, along with decrease in O$_2^-$ levels in HTFL-PAEC after Sep treatment, suggests that eNOS is recoupled.

Our observation that HTFL-PAEC have a lower eNOS dimer-to-monomer ratio than NFL-PAEC and that Sep treatment increases the ratios by almost 16 times suggests that decreases in GCH-1 expression and BH4 levels play an important role in the uncoupling of eNOS activity in HTFL-PAEC. This is further supported by the finding that the association of eNOS with hsp90 in HTFL-PAEC increased nearly fourfold after Sep treatment. Our results show that Sep increases coupled eNOS activity at two levels: increase in hsp90-eNOS interaction and increased eNOS-hsp90 association. Whether these two events are mechanistically related requires further investigation.

In conclusion, our data suggest that Sep improves the angiogenic potential of HTFL-PAEC through enhanced NO production. Our data suggest that uncoupled eNOS activity resulting from decreased BH4 level plays an important role in the mechanisms by which pulmonary hypertension impairs angiogenesis.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES
24. Kunduri GG, Ou J, Shi Y, Pritchard KA Jr. Decreased association of HSP90 impairs endothelial nitric oxide synthase in fetal lambs with...


