Role of platelet-activating factor in pulmonary vascular remodeling associated with chronic high altitude hypoxia in ovine fetal lambs

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Bixby CE, Ibe BO, Abdallah MF, Zhou W, Hislop AA, Longo LD, Raj JU. Role of platelet-activating factor in pulmonary vascular remodeling associated with chronic high altitude hypoxia in ovine fetal lambs. Am J Physiol Lung Cell Mol Physiol 293: L1475–L1482, 2007. First published October 19, 2007; doi:10.1152/ajplung.00089.2007.—Platelet-activating factor (PAF) is implicated in pathogenesis of chronic hypoxia-induced pulmonary hypertension in some animal models and in neonates. Effects of chronic hypoxia on PAF receptor (PAF-R) system in fetal pulmonary vasculature are unknown. We investigated the effect of chronic high altitude hypoxia (HAH) in fetal lambs [pregnant ewes were kept at 3,801 m (12,470 ft) altitude from ∼35 to 145 days gestation] on PAF-R-mediated effects in the pulmonary vasculature. Age-matched controls were kept at sea level. Intrapulmonary arteries were isolated, and smooth muscle cells (SMC-PA) were cultured from HAH and control fetuses. To determine presence of pulmonary vascular remodeling, lung tissue sections were subjected to morphometric analysis. Percentage medial wall thickness was significantly increased (P < 0.05) in arteries at all levels in the HAH lambs. PAF-R protein expression studied by immunocytochemistry and Western blot analysis on lung tissue SMC-PA demonstrated greater PAF-R expression in HAH lambs. PAF-R binding (femtomoles per 106 cells) in HAH SMC-PA was 90.3 ± 4.08 and 66% greater than 54.3 ± 4.9 in control SMC-PA. Pulmonary arteries from HAH fetuses synthesized >3-fold PAF than vessels from controls. Compared with controls SMC-PA of HAH lambs demonstrated 139% and 40% greater proliferation in 10% FBS alone and with 10 nM PAF, respectively. Our data demonstrate that exposure of ovine fetuses to HAH will result in significant upregulation of PAF synthesis, PAF-R expression, and PAF-R-mediated effects in pulmonary arteries. These findings suggest that increased PAF-R protein expression and increased PAF-R binding contribute to pulmonary vascular remodeling in these animals and may predispose them to persistent pulmonary hypertension after birth.

platelet-activating factor receptor binding; pulmonary arteries; smooth muscle; cell proliferation

PLATELET-ACTIVATING factor (1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine; PAF) is a phospholipid with diverse biological actions (21). High PAF plasma levels have been reported in newborns with persistent pulmonary hypertension of the newborn (PPHN) with a fall in PAF levels as they improved clinically (6). PAF has also been implicated in chronic hypoxia-induced pulmonary hypertension (PH) in adult rats (7, 34). In those studies, rats placed in chronic hypoxia developed PH and right ventricular hypertrophy (RVH) as well as a significant increase in PAF in the lung (7). Furthermore, PAF receptor (PAF-R) antagonists attenuated hypoxia-induced PH and pulmonary vascular remodeling (34), indicating a PAF-R-mediated mechanism.

In previous reports, we (13, 18, 19) demonstrated, by using specific PAF-R antagonists infused into fetal lambs in vivo, that PAF acting via its specific receptors contributes significantly to maintenance of high vasomotor tone in the pulmonary circulation in utero. We have demonstrated that acute hypoxia increases PAF synthesis in fetal lung vascular smooth muscle cells (SMC) (14, 16); hence, we speculate that in the hypoxic environment of fetal lungs, more PAF may be available for binding to its receptor. We (19) have also reported that PAF-R gene mRNA expression and PAF-R density are high in fetal lungs, both of which decrease within 2 h after birth, returning to a new intermediate level after a few weeks of life. Furthermore, we found that acute hypoxia upregulates PAF-R binding as well as PAF-R protein levels in pulmonary vascular SMC of fetal lambs (15). These findings support a unique role for PAF as an important endogenous mediator of high pulmonary vasomotor tone in the fetus.

In this report, we tested the hypothesis that chronic in utero hypoxia upregulates PAF and PAF-R protein in the pulmonary vasculature of the fetal lamb. We determined the effect of chronic in utero hypoxia induced by high altitude (HAH) on the vascular structure by morphometric analysis of blood vessels in fixed lung sections from chronically hypoxic and control fetal lambs. Additionally, we determined the effect of HAH on PAF synthesis in pulmonary arteries, PAF-R protein expression (by immunocytochemistry and Western blotting) in pulmonary artery SMC (SMC-PA), PAF-R binding to the vascular structure by morphometric analysis of blood vessels in fixed lung sections from chronically hypoxic and control fetal lambs. Additionally, we determined the effect of HAH on PAF synthesis in pulmonary arteries, PAF-R protein expression (by immunocytochemistry and Western blotting) in pulmonary artery SMC (SMC-PA), PAF-R binding to the SMC, and the effect of PAF on SMC proliferation.

MATERIALS AND METHODS

Materials

Pregnant ewes were purchased from Nebekar Farms (Los Angeles, CA). Authentic standards of PAF (hexadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine; C16-PAF) were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Tritiated PAF and thymidine were purchased from PerkinElmer (Wellesley, MA). Monoclonal human anti-PAF-R antibody was purchased from Cayman Chemical (Ann Arbor, MI). FITC-conjugated goat anti-rabbit IgG and SMC-specific monoclonal antibody and 3,3′-diaminobenzidine (DAB) were purchased from Sigma (St. Louis, MO). Confocal micrographs were purchased from Sigma (St. Louis, MO). Confocal micrographs were purchased from Sigma (St. Louis, MO). Confocal micrographs were purchased from Sigma (St. Louis, MO). Confocal micrographs were purchased from Sigma (St. Louis, MO). Confocal micrographs were purchased from Sigma (St. Louis, MO). Confocal micrographs were

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obtained on Leica TCS SP confocal microscope (Leica Microsystems). Antibody to smooth muscle-specific α-actin isoform was purchased from Sigma (Poole, United Kingdom). Biotin-conjugated sheep anti-mouse secondary antibody and streptavidin biotinylated horseradish peroxidase (HRP) complex were purchased from Amersham.

Methods

Study groups. Fetal lungs from the following groups of pregnant ewes were used to prepare fixed lung tissue sections for morphometry, lung tissue membrane preparations, and culture of SMC-PA.

HAH group. Pregnant ewes were kept at an altitude of 3,801 m (12,470 ft) from day 35 to day 145 of gestation (term being 150 days). The ewes were brought to sea level and killed the following day. The fetuses were then removed and their lungs harvested for use in our studies.

Control group. Pregnant ewes were kept at sea level for their entire gestation and killed at comparable gestational age to the HAH group. All cell culture studies were conducted at 37°C in incubators aerated with 5% CO₂ in air.

The studies were approved by the Institutional Review Board and the Animal Care and Use Committee of Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center and the Institutional Review Board and the Animal Care and Use Committee of Loma Linda University.

Morphometry

Immunohistochemistry. Four-micrometer sections were made from formalin-fixed lung tissue and stained with antibody to smooth muscle-specific α-actin isoform (Sigma). Heat antigen retrieval was performed, and endogenous peroxidase activity and nonspecific antibody binding were blocked. Sections were incubated with primary antibody for 1 h at room temperature for α-actin (1:3,000 dilution) followed by the biotin-conjugated sheep anti-mouse secondary antibody. The streptavidin biotinylated HRP complex was incubated for 30 min at room temperature with DAB. The sections were lightly counterstained with hematoxylin.

Measurement of muscle in peripheral arteries. Color images from α-actin-stained slides of HAH and control animals were obtained with a digital camera (Zeiss AxioCam; Imaging Associates, Thame, Oxfordshire, United Kingdom). Analysis was made using Openlab v.3.15 software (Improvision, Coventry, United Kingdom). Muscular arteries accompanying specific airways (small bronchioli, terminal bronchioli, respiratory bronchioli, and alveolar ducts) were studied in each group (at least 10 arteries at each level for each animal). The external diameter and lumen plus endothelium diameter were measured. Medial wall thickness was expressed as a percentage of the external diameter. The total area of the artery (including the lumen, endothelium, and media) and the area of the lumen and endothelium were measured. The medial wall area was calculated and expressed as percentage of the total area. A mean value for each airway level in each group was calculated for percentage medial wall thickness, as there was no statistical difference between animals in the same group (27).

Studies With SMC-PA

Preparation of SMC. Intrapulmonary arteries, 2nd to 6th generation, were dissected from the HAH and control groups. SMC were harvested under sterile conditions as previously reported (15, 16, 35). Cells were used at 3rd to 7th passage. SMC identity was confirmed with α-SMC actin (α-SMA)-specific monoclonal antibody (Calbiochem). SMC cultures were devoid of endothelial cells and fibroblasts.

Western Blotting for PAF-R Protein Expression

Preparation of membrane protein from whole lung tissues. Lung parenchymal tissue from the HAH and control fetal lambs were washed, quickly frozen in liquid nitrogen, and then homogenized separately at 4°C in 9 volumes of buffer per gram of lung tissue (19, 37). The homogenization buffer consisted of 50 mM Tris buffer, pH 7.2, containing 0.1 mM PMSF (Sigma-Aldrich). The membrane layer was isolated by centrifuging first at 3,000 rpm for 10 min to rid the homogenate of tissue debris, followed by centrifugation of the supernatant at 40,000 g at 4°C for 20 min. The pellet was resuspended in 50 mM Tris buffer containing 0.1 mM PMSF, 0.1 µg/ml leupeptin, and 1 µg/ml pepstatin (Sigma-Aldrich) at a ratio of 1 volume buffer per gram of original tissue weight. Protein concentration of the lung membrane suspension was determined by the method of Bradford (4) using BSA as the standard. Proteins were frozen in liquid nitrogen in 0.5-ml aliquots and stored at −80°C.

The membrane proteins used in PAF-R binding assays were resuspended in the 50 mM Tris buffer containing 5 mM MgCl₂, 0.1 mM PMSF, 125 mM choline chloride, 0.25% BSA, as well as 1.1 M sucrose.

Preparation of proteins from SMC. Proteins were prepared from subconfluent SMC-PA grown on 150-mm Petri dishes. Cells were first washed twice with Ca²⁺/Mg²⁺-free PBS and treated with a hypotonic lysis buffer to lyse the cells as previously reported (15). Cells were then scraped and collected in a centrifuge vial to be sonicated for 60 s over ice. The cell lysate was centrifuged at 14,000 rpm for 5 min on refrigerated Eppendorf centrifuge. The supernatant was collected, and the protein concentration was quantified by the method of Bradford (4) using BSA as the standard.

Preparation of subcellular protein fractions from SMC-PA. The cell nuclear and membrane fractions were prepared by centrifuging the lysed cells at 500 g for 10 min on a refrigerated centrifuge. The cell pellet (nuclear fraction) was resuspended in the lysis buffer for use in Western blotting of PAF-R in the nuclear fraction. The 500 g supernatant was spun at 100,000 g for 1 h to harvest the membrane and cytosolic fractions. The 100,000 g pellet (the membrane) was resuspended in the lysis buffer. PAF-R expression was studied in nuclear and membrane fractions by Western blotting. PAF-R binding in nuclear and membrane fractions was also determined.

The nuclear and membrane fractions used in PAF-R binding assays were resuspended in the lysis buffer containing 1.1 M sucrose, 125 mM choline chloride, 5 mM MgCl₂, and 0.25% BSA.

SDS-PAGE electrophoresis. After loading 5 µg of protein for each sample, were electrophoresed for 1 h at 200 V on a 4–12% Tris-glycine gradient gels (C Bamrck) along with Precision Plus Protein Standards (Bio-Rad). Proteins were transferred to nitrocellulose membrane via Trans-Blot (Bio-Rad) at 90 V for 2 h. Proteins were then blocked with 5% nonfat dry milk in 1% Tween 20-TBS (1%T-TBS) overnight. Blots were incubated for 1 h with 1:500 dilution of human PAF-R antibody (monoclonal, Cayman), washed with 1%T-TBS, incubated for 1 h with anti-mouse secondary antibody (1:1,000 dilution, Cell Signaling), and followed by washes with 1%T-TBS and finally 1% TBS. The signals were developed for 5 min using Pierce SuperSignal West Pico Chemiluminescent Substrate detection kit and then exposed to X-ray film. PAF-R protein band was quantified using UN-SCAN-IT digitizing gel software (v.5.1). Membranes were then stripped with Restore Western Blot Stripping Buffer (Pierce) and reprobed for actin (1:10,000 dilution, Calbiochem) followed by anti-mouse secondary antibody (Amersham) in the same fashion as described for PAF-R. The blots were developed for 1 s, and the actin blot was quantified. PAF-R blot density was expressed as a ratio of the actin blot density.

PAF-R Binding

PAF-R binding assay in lung tissue membrane proteins. PAF-R binding assay was performed at 30°C for 1 h on 100 µg of lung tissue protein per milliliter of assay volume. The assay buffer was a 50 mM Tris buffer, pH 7.2, containing 0.1 mM PMSF, 125 mM choline chloride, and 125 mM choline chloride, and 0.25% BSA, as well as 1.1 M sucrose.
chloride, 5 mM magnesium chloride, 1.1 M sucrose, 0.25% BSA, and [3H]acetyl-C16-PAF (H-PAF). The concentrations of PAF used were based on known physiological levels of PAF (13). Receptor-bound H-PAF was extracted on GF/C filters membranes (Whatman, Boston, MA) using an in-line vacuum system. Receptor-bound PAF was quantified radiometrically. PAF binding to its membrane receptors is expressed as femtomoles per milligram of protein.

**Assay of PAF-R binding on SMC-PA.** Binding assays were done on adherent SMC-PA under normoxic conditions. Thus the SMC-PA of control and HAH fetal lambs were not subjected to further in vitro hypoxic conditions. Binding assay was done as previously reported (15, 19, 24). Briefly, 3rd and 6th passage subconfluent cells in six-well culture plates were washed with Ca2+- and Mg2+-free PBS and then treated with 10 nM [3H]-PAF and incubated in normoxia for 30 min at 37°C. We used 10 nM [3H]-PAF, which is within the physiological range of PAF concentration, in the binding assays. The PAF-R binding assay buffer was DMEM containing 0.1% BSA. Reaction was stopped by placing cells on ice, aspirating the media, and washing with ice-cold Ca2+- and Mg2+-free PBS. A solution of 157 mM NaCl and 5 mM EDTA was added to each well and incubated on ice for 30 min. Cells were then scraped, and receptor-bound [3H]-PAF was extracted on Whatman GF/C filters using an in-line vacuum system. In experiments to study the specificity of PAF-R binding, the adherent cells were preincubated for 30 min at 37°C with 1 μM PAF-R antagonist CV-6209, and then 10 nM [3H]-PAF was added and incubated for 30 min more. Receptor-bound PAF in the inhibitor and noninhibitor studies was quantified radiometrically and expressed as femtomoles per 106 cells.

To study PAF binding to its receptors in the proteins from the subcellular fractions, nuclear and membrane proteins of the SMC-PA of control and HAH fetal lambs were subjected to a similar receptor binding protocol as in the studies with the lung tissue membrane proteins described above.

**PAF synthesis by pulmonary artery membrane proteins of control and HAH lambs.** PAF synthesis was studied by measuring the activity of lyso-PAF acetyl-S-CoA acetyltransferase present in membrane proteins of intrapulmonary arteries of the control and HAH lambs. For each membrane protein, 25 μM aliquot of lyso-PAF and 250 μM [3H]acetil-S-CoA were placed in a polypropylene tube and warmed to 37°C in a shaker bath. The reaction was initiated by adding 100 μg of membrane protein of each vessel type for a total volume of 1 ml of a medium containing 0.025% BSA, and incubating the mixture at 37°C for 10 min. After incubation, H-PAF synthesized was extracted and subjected to TLC purification (14). Thin layer chromatogram corresponding to authentic PAF in retention time (Rf value) was purified further by HPLC. HPLC fractions eluting with authentic PAF standard were collected and quantified for PAF radioactivity (14).

**Cell proliferation.** Cell proliferation was measured by [3H]thymidine incorporation into cells. SMC-PA (3rd to 6th passage) was cultured in six-well plates until they attained 50–60% confluency. Growth was arrested in normoxia for 72 h by incubating in 0.1% FBS in DMEM followed by incubation for another 24 h in 10% FBS in normoxia with 1 μCi [3H]thymidine (Perkins-Elmer) with or without 10 nM nonradioabeled PAF (Biomol Research Laboratories). Studies with 1 μM specific PAF-R antagonist CV-6209 was used to test the specificity of PAF-R-mediated cell proliferation. After 24 h in culture, unincorporated radioactivity was aspirated, and each well was washed twice with ice-cold PBS, followed by wash with 5% trichloroacetic acid. Cells were then treated with 0.5 M NaOH, and radioactivity of cell suspension was measured by scintillation spectrometry (LS 6500 Scintillation Counter; Beckman Coulter, Fullerton, CA). Direct cell count was performed by hemocytometer to corroborate data from [3H]thymidine incorporation.

**Data Analysis**

All numerical data are presented as means ± SE and analyzed statistically by two-tailed Student’s t-test. For multiple comparisons, an ANOVA was used with Bonferroni post hoc test. P < 0.05 was considered statistically significant.

**RESULTS**

**Birth Weight and Sex of Lambs**

Male and female fetuses were studied. Birth weights of control (n = 15) and HAH (n = 13) fetuses were similar, 8.3 ± 0.2 lb vs. 8.6 ± 0.6 lb., respectively.

**Chronic HAH In Utero Induces Remodeling of Pulmonary Arteries in Term Ovine Fetuses**

Figure 1 shows the percent medial wall thickness for the pulmonary arteries accompanying specific airways for the control (n = 5) and high altitude fetuses (n = 5). We found a significant increase in the percent medial wall thickness in the high altitude fetal pulmonary arteries compared with the controls. Of note, the greatest difference was seen in the small pulmonary arteries associated with alveolar ducts.

**PAF-R Studies**

Western blot analysis shows increased PAF-R protein in lung tissue membrane and SMC-PA of HAH lambs. Figure 2 shows PAF-R protein expression by lung tissue and SMC-PA of HAH and control fetal lambs studied by Western blotting. Figure 2A shows PAF-R protein expression measured in lung tissue membrane proteins and SMC-PA of control and HAH lambs expressed as ratio of α-SMA (actin) internal standard. Lung tissue membranes of the HAH lamb expressed 40% more PAF-R protein than lung tissue from control lambs. Figure 2B shows PAF-R protein expression measured in protein from cultured SMC-PA of control and HAH lambs and expressed as a ratio of actin internal standard. Compared with expression of the SMC-PA of the control lambs, PAF-R protein expression by SMC-PA of the HAH fetal lambs was twofold greater. Figure 2C shows PAF-R protein expression in nuclear and membrane fractions of control and HAH lambs expressed as ratio of GAPDH internal standard. PAF-R protein was...
detected in nuclear and membrane fractions of the two groups of lambs, however, expression was greater in nuclear and membranes fractions of SMC-PA of the HAH lambs.

**Study of PAF Binding to its Receptors**

PAF-R binding in lung membranes of chronic HAH fetal lambs is greater than binding in lung membranes of control lambs. Figure 3A shows PAF binding to its receptors in lung membrane protein of control and HAH fetal lambs. With 0.8 nM PAF, PAF-R binding (femtomoles per milligram of protein) in lung membrane of controls was 1,263 ± 94, which is different from 1,574 ± 69 in HAH. Binding of PAF to its receptors in lung membranes of the HAH fetal lambs was greater than binding in lung membranes of control lambs at physiological concentrations of PAF studied. For instance, at the physiological PAF concentration of 10 nM, binding in membrane proteins from HAH lambs was 23% greater, and the difference increased to 30% with 16 nM PAF. Interestingly, 10 nM PAF produced a greater than 10-fold increase in PAF binding in membranes of the HAH and control lambs compared with 0.8 nM PAF. The increase in binding from 10 to 16 nM PAF treatment was not as dramatic in both groups.

PAF-R binding to adherent SMC-PA of chronic HAH fetal lambs is greater than binding to adherent SMC-PA of control fetal lambs. Cell passage from 3 to 7 did not affect PAF binding. Figure 3B shows PAF binding to its receptors in SMC-PA of HAH lambs compared with binding in SMC-PA of controls. PAF binding (femtomoles per 10^6 cells) to receptors in the HAH fetal lambs was 41% greater than binding to SMC-PA of the control fetal lambs. In both control and HAH cells, there was no difference in PAF-R binding between the 3rd and the 6th passage cells. Also, in both cells types, the specific PAF-R antagonist CV-6209 attenuated PAF-R binding, indicating specific involvement of PAF receptors. When we examined PAF binding to its receptor in specific cell compartments purified by differential centrifugation (Fig. 3C), we found that PAF-R binding in membrane fractions of cells from the HAH lambs was fourfold greater than binding in the membrane fraction of the controls. Also, binding in nuclear fraction of the HAH cells was twofold greater than binding in nuclear fraction of control cells. Thus there is significantly greater PAF-R protein expres-

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**Fig. 2.** A–C: platelet-activating factor (PAF) receptor (PAF-R) protein expression by lung tissue and pulmonary artery smooth muscle cell (SMC-PA) of HAH and control fetal lambs. The numerical data are presented as a ratio of density of the PAF-R protein to the blot density of total actin standard (A and B), or as a ratio of GAPDH internal standard (C). For all the panels, the top is a representative blot of 4 different animal studies, and numerical data are means ± SE, n = 4. In A, membrane protein from lung tissue of control and HAH fetal lambs was probed for PAF-R expression and normalized to actin internal standard. The legend on the top is as presented. In B, protein was prepared from confluent SMC-PA of control and HAH lambs, and PAF-R protein expression was studied by Western blotting. On the top, the left lane shows PAF-R protein expression by SMC-PA of control fetal lambs, and the right lane shows receptor expression by SMC-PA of HAH fetal lambs. In C, nuclear and membrane proteins prepared from confluent SMC-PA of control and HAH lambs were probed for PAF-R protein expression and normalized to GAPDH internal standard. Top: lanes 1 and 3 are PAF-R blots of nuclear fractions, and lanes 2 and 4 are PAF-R blots of the 100,000 g membrane fractions of controls and HAH fetal lambs, respectively. *P < 0.05 vs. control fetal lambs or nuclear fraction; #P < 0.05 vs. nuclear fraction or membrane of control fetal lambs.
sion as well as PAF binding to its receptor in lung tissue and SMC-PA of HAH fetal lambs.

PAF synthesis by intrapulmonary arteries of HAH fetal lambs is greater than the synthesis by intrapulmonary arteries of control fetal lambs. Figure 4 shows PAF synthesis by membrane proteins of intrapulmonary arteries of control and HAH fetal lambs. The lyso-PAF acetyl-S-CoA acetyltransferase present in the membranes of pulmonary arteries synthesized PAF from acetyl CoA when the membrane proteins were fed lyso-PAF. PAF synthesis by proteins from HAH fetal lambs was more than threefold greater than the proteins from intrapulmonary arteries of control fetal lambs.

**SMC-PA of chronic HAH fetal lambs exhibit greater proliferative potential than SMC-PA of control lambs and PAF augments the proliferative effect.** Figure 5 shows effect of PAF on proliferation of SMC-PA of control and HAH fetal lambs. In SMC-PA of control lambs, treatment with 10 nM PAF evoked ~2-fold increase in proliferation compared with effect of 10% FBS alone. With SMC-PA of the HAH fetal lambs, 10% FBS produced twofold greater cell proliferation than the effect of 10% FBS alone in SMC-PA of control lambs. Treatment of the SMC-PA of the HAH fetal lambs with 10 nM PAF produced 60% greater cell proliferation compared with effect of 10% FBS alone. This is a greater than 60% increase in proliferation compared with the effect of 10 nM PAF on SMC-PA of control fetal lambs. Also, 10% FBS produced as much proliferation of the SMC-PA of the HAH lambs compared with effect of 10 nM PAF on the SMC-PA of the control lambs. In both SMC-PA of control and HAH lambs, PAF-
stimulated cell proliferation was inhibited by 1 μM specific PAF-R antagonist CV-6209. Therefore, SMC-PA of the HAH fetal lambs exhibited greater proliferation than cells of the control lambs whether in baseline or after treatment with PAF and proliferation occurred via PAF-R-specific pathway.

DISCUSSION

In this study, we investigated the effect of fetal exposure to chronic HAH on the pulmonary vascular bed of the fetus and the role of PAF in the observed changes. We found that lungs and SMC-PA from HAH fetal lambs expressed greater PAF-R protein than the controls. Pulmonary vascular changes in PPHN have been shown to include increased muscularity of arteries distally, with medial thickening and a decreased number of peripheral arterioles (1, 12, 28). In a previous report using a chronic hypobaric hypoxia model in adult rats, Ono et al. (34) described similar pulmonary vascular remodeling with increased pulmonary artery pressure, RVH, and polycythemia. Treatment of the rats with a PAF-R antagonist, WEB 2170, in the hypobaric hypoxia rat model decreased the pulmonary vascular remodeling, PH, and RVH, suggesting an association between PAF, its receptor, and the observed pulmonary vascular abnormalities (34).

In the current study, pulmonary arteries from ovine fetuses were evaluated for morphometric changes consistent with the pulmonary vascular remodeling seen in PPHN and the above rat model. Morphometric analysis of the pulmonary arteries demonstrated increased muscularity of the HAH fetal lambs at the alveolar level and increased medial wall thickness, suggesting a decrease in luminal diameter of the pulmonary artery. These findings are consistent with the hallmark pulmonary vascular remodeling seen in human newborns with PPHN and in hypoxia-induced PH in animal models (1, 10, 12, 26, 34, 39). Although we do not have physiological data in our model documenting PH, the similarity in remodeling seen in our HAH fetuses suggests that these animals either have or have the potential to develop PH if the fetuses were allowed to undergo transition from fetal to newborn circulation.

In addition to the above pulmonary vascular remodeling, PH is also associated with polycythemia and RVH. Previous works with this same animal model (22, 23) have shown that maternal arterial PO2 decreases to ~60 Torr while at the high altitude. These same ewes were brought to sea level, and hypoxemia was established by administering nitrogen to maintain arterial PO2 at ~60 Torr. Fetal PO2 was then measured, demonstrating a significantly reduced fetal PO2 of 19.3 ± 0.8 compared with 23.3 ± 0.5 Torr for control animals. Thus, although fetal environment in utero is hypoxic, exposure of the pregnant ewes to high altitude further decreased the fetal PO2 level. This result is consistent with changes seen in animal models with PH (1) and chronic in utero hypoxia (9), in human newborns born at high altitude (2, 32, 33), and in some neonates with PH (38). The right ventricle (RV) size in the HAH fetal lamb has been previously reported and was found to be the same or slightly smaller in size compared with control animals (5, 25). Human neonates born at high altitude have been shown to have a prolonged RV predominance during infancy suggestive of elevated pulmonary artery (PA) pressures but not necessarily RVH at birth (30, 33). These infants born at high altitude were found to have an ~100-fold increase of “impaired cardiopulmonary transition” compared with sea level controls (30, 31, 33). Of note, infants born to mothers with less high altitude exposure before pregnancy had greater polycythemia, more prolonged RV predominance, even into the latter part of infancy, and lower oxygen saturations (33). RVH has been described in newborns with PPHN (12, 38) and in fetal sheep with late gestation in utero compression of the ductus arteriosus (1). In contrast to the ductal compression model and the neonate with PPHN, our HAH model has not undergone transition to newborn life nor has the RV had to pump the entire cardiac output through the pulmonary vascular bed. There may indeed be elevated PA pressures in our HAH fetal lambs, but the ductus arteriosus may serve as a pop-off while in utero, making comparison inappropriate. Additionally, the previously described decrease in RV stroke volume and RV output in our HAH fetal lamb model may also reflect a difference in the ability of the fetal ovine RV to respond to chronically decreased PaO2 (23). A decreased response by the fetal RV to acutely superimposed hypoxia in these HAH animals has also been reported (23). Previous morphometric analysis of the RV of the HAH fetal heart demonstrated increased fiber cross-sectional area, possibly denoting early changes toward RVH (25). Overall, our morphometric findings of the HAH fetal lamb are consistent with PH, and the additional data about this animal model support the presence or potential for PH.

Chronic HAH and PAF-R Protein Expression

The involvement of PAF and PAF-R in PH and pulmonary vascular remodeling has been demonstrated by use of a PAF-R antagonist in a rat model of hypobaric hypoxia (34). Since the HAH fetuses exhibited characteristics consistent with pulmonary vascular remodeling, we investigated the expression of PAF-R protein to elucidate any relationship between the observed remodeling and PAF. PAF-R is a member of G protein-coupled receptors, which is a family of proteins with seven transmembrane loops (21, 29, 36). PAF-Rs are distributed throughout many tissues including the pulmonary vascular bed.
We (19) have previously shown that PAF-R protein expression is higher in fetal lamb lungs compared with the lungs of the newborn lamb. We (15) have shown that hypoxia upregulates PAF-R protein expression in the pulmonary vascular SMC of the ovine fetus studied in vitro. In this study, PAF-R protein expression by lung tissues from the control and HAH lambs was assessed by Western blotting. Lungs of the HAH fetal lambs showed significantly higher expression of PAF-R protein than the controls. Furthermore, Western blotting of PAF-R protein expression in SMC-PA of control lambs and Western blotting of proteins from SMC-PA of control lambs showed significantly higher expression of PAF-R protein in nuclear and membrane fractions. These findings support a role for PAF in the pulmonary vascular remodeling observed in pulmonary arteries of the HAH fetuses. Additionally, the greater nuclear translocation of PAF-R to nuclear compartment of SMC-PA of HAH fetal lambs may indicate a unique mechanism by which PAF-R is activated by HAH to induce cell growth and subsequent vessel wall thickening. When SMC of control fetal lambs were exposed to in vitro hypoxia for 2 h, PAF-R colocalized in the nuclear compartment of the cells studied, which was not observed in cells exposed to normal oxygen tension (Ibe BO, unpublished observation). The physiological implications of this apparent hypoxia-induced PAF-R nuclear colocalization is not clear at this time, but it does suggest that the observed greater nuclear localization of PAF-R protein in SMC-PA of HAH fetal lambs is a consequence of the in utero exposure of the fetus to chronic hypoxia. The findings of increased PAF-R protein expression in lungs of HAH fetal lambs strongly suggest a role for PAF and PAF-R-mediated effects in the observed medial wall thickening of pulmonary arteries seen in fetal lambs exposed to chronic in utero HAH.

**Chronic HAH and PAF-R Binding**

We (19) have previously shown that PAF-R binding is high in fetal ovine lungs, and the binding falls dramatically immediately after birth. In this study, PAF-R binding in the lung parenchymal membrane of the HAH fetal lambs was significantly greater compared with the binding in lung membranes of control fetal lambs. The result of these binding studies shows that the receptors that were characterized in lung tissue by Western blotting are functional PAF receptors. On further investigation of PAF-R binding in SMC-PA, we also found significantly greater binding in cells of the HAH fetal lambs. PAF binding to its receptors was not influenced by cell passage number. This strongly suggests that the observed difference in binding between control and HAH cells is due to the cell origin, i.e., cells from HAH fetal lambs rather than the cell passage. This information, together with increased PAF binding to the nuclear and membrane proteins, further supports a role for PAF in the observed pulmonary vascular remodeling. Interestingly, we found that PAF synthetic activity by membrane proteins from intrapulmonary arteries of the HAH lambs was more than threefold greater than the synthetic activity of the control lambs. PAF is an autacoid with significant vasoactivity in the pulmonary circulation of the fetus (13). Its synthesis occurs locally at site of action. Thus, with respect to the HAH fetal lambs in vitro, greater PAF synthesis by the arteries, coupled to the greater PAF-R protein expression, will predispose the smooth muscle to greater PAF binding and greater PAF-R-mediated effects.

**Effect of PAF on SMC-PA Proliferation**

SMC-PA from humans and other animal models have been shown to proliferate in response to a hypoxic environment (3, 8, 11). Data from studies in a bovine model of hypobaric hypoxia-induced PH suggest that structural remodeling in PH may be due partly to increased proliferation in the adventitia and media (3, 39). Fetal ovine SMC displayed increased proliferation in response to PAF alone, and the combination of PAF and hypoxia leads to a further increase in proliferation (17). In the current study, SMC-PA from HAH fetuses displayed greater proliferative potential compared with cells from control fetal lambs. Exposure of SMC-PA to PAF resulted in an increase in proliferation in cells from HAH and control fetal lambs, however, the proliferation of cells from HAH fetal lambs exposed to PAF was 65% greater than control cells exposed to PAF. This finding of increased proliferation with exposure to PAF, along with the increase in PAF synthesis, increase in PAF-R protein expression, as well as increase in PAF-R binding in nuclear fraction seen in the lungs of the HAH fetal lambs, further demonstrates a role for PAF as a mitogen involved in the induction of pulmonary vascular remodeling.

In summary, we found pulmonary arterial remodeling in fetal lambs exposed to chronic HAH. We also found increased PAF synthesis in pulmonary arteries, increased PAF-R protein expression, increased PAF-R binding, and an increase in PAF-induced SMC-PA proliferation. This report is the first to describe a specific role for PAF in the pulmonary vascular remodeling of fetal lambs exposed to HAH in utero. Our data strongly suggest that in vivo, increased pulmonary vascular PAF synthesis together with increased PAF-R protein expression will allow more PAF to bind to its receptor to evoke a sequence of events that includes exaggerated cell growth that will ultimately result in pulmonary vascular remodeling. We speculate that with an abnormal state of hypoxia in utero, PAF-induced pulmonary vascular remodeling may result in persistence of high fetal pulmonary vasomotor resistance postnatally and subsequently to clinical PPHN.

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