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## Role of Platelet-Activating Factor and Hypoxia in Persistent Pulmonary Hypertension of the Newborn — Studies with Perinatal Pulmonary Vascular Smooth Muscle Cells

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Additional information is available at the end of the chapter

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### Abstract

Platelet-activating factor (PAF) plays an important physiological role of maintaining a high vasomotor tone in fetal pulmonary circulation. At birth, endogenous vasodilators such as nitric oxide and prostacyclin are released and facilitate pulmonary vasodilation via cAMP-dependent protein kinase (cAMP/PKA) and cGMP-dependent protein kinase (cGMP/PKG) pathways. Interaction between the cyclic nucleotides and PAF receptor (PAFR)-mediated responses in pulmonary arterial smooth muscle is not well understood. To further understand the interactions of PAF-PAFR pathway and the cyclic nucleotides in ovine fetal pulmonary arterial smooth muscle cells (FPASMC), effects of cAMP and cGMP on PAFR-mediated responses in pulmonary arterial smooth muscle cells (PASMC) were studied. Ovine FPASMC were incubated with 10 $\mu$ M cAMP or cGMP in normoxia (5% CO<sub>2</sub> in air, pO<sub>2</sub>~100 Torr) or hypoxia (2% O<sub>2</sub>, 5% CO<sub>2</sub>, pO<sub>2</sub>~30-40 Torr). Proteins were prepared and subjected to Western blotting. Effect of cell permeable cAMP and cGMP on PAFR binding was also studied and effect of cAMP on cell proliferation was also studied by RNAi to PKA-C $\alpha$ . cAMP and cGMP significantly decreased PAFR binding and protein expression in normoxia and hypoxia, more so in hypoxia, when PAFR expression was usually high. PKA-C $\alpha$  siRNA demonstrated that inhibition of PAFR-mediated responses by the cyclic nucleotides occurred through PKA. These data suggest that the normally high levels

of cyclic nucleotides in the normoxic newborn pulmonary circulation assist in the downregulation of postnatal PAFR-mediated responses and that under hypoxic conditions, increasing the levels of cyclic nucleotides will abrogate PAF-mediated vasoconstriction thereby ameliorating PAF-induced persistent pulmonary hypertension of the newborn.

**Keywords:** Pulmonary artery, PAFR binding, cyclic nucleotides, siRNA

## 1. Introduction

Platelet-activating factor (PAF) is an endogenous phospholipid which evokes a wide range of biological activities, such as vasoconstriction and systemic hypotension [1], mainly under pathophysiological conditions. The discovery of PAF, its cellular origin, and biological actions were first reported by Benveniste and Associates [2, 3]. Following these reports, investigations of the physiological effects of PAF involved its roles in fetal lung maturation and lung function [4-9], and its role in reproduction where it is involved in implantation of embryos, among other effects [10, 11]. PAF produces a myriad pathological effects *in vivo*, including platelet aggregation [12-14], mediation of immune response and bronchoconstriction [15, 16], and smooth muscle contraction [17-21], which hinges on its role as an inflammatory mediator and vasoconstrictor [17]. In the fetus, PAF plays an important physiological role in maintaining a high level of vasomotor tone in the pulmonary circulation [22]. Therefore, the high PAF receptor (PAFR) binding in fetal lamb lungs supports the existence of a high level of pulmonary vasomotor tone *in utero* [23]. On the other hand, in lungs of the newborn lamb, PAFR binding and receptor mRNA expression are low, suggesting a down regulation of PAFR-mediated effects *in vivo* [22, 23].

PAF acts by binding to its Gq protein isoform of G protein-coupled receptors, which is a seven transmembrane receptor [24]. Activation of G protein-coupled receptors by an agonist results in activation of signal transduction pathways [25], which may involve recruitment of intracellular second messengers such as cAMP, cGMP, inositol 1,4,5-triphosphate (IP<sub>3</sub>), and calcium [26, 27]. cAMP and cGMP act via their endogenous receptors, cAMP-dependent protein kinase (cAMP/PKA) and cGMP-dependent protein kinase (cGMP/PKG), respectively, to elicit relaxation of smooth muscle, and cAMP and cGMP mediate relaxation of pulmonary vessels, but cGMP has been shown to be more effective than cAMP in producing relaxation of perinatal ovine pulmonary vessels [28, 29]. Acute hypoxia upregulates PAFR-mediated intracellular signaling in fetal ovine pulmonary vascular smooth muscle [30]. Chronic hypoxia in the perinatal period may result in abnormal upregulation of PAFR protein expression, PAFR binding, and PAFR-mediated cell signaling, leading to increased pulmonary vasomotor tone and vascular remodeling, a key event in the onset of clinical disorders such as persistent pulmonary hypertension of the newborn (PPHN) [31].

We are interested in understanding the mechanisms of pulmonary vascular relaxation at birth. Our primary hypothesis is that with oxygenation at birth and the increased production of

cAMP and cGMP in pulmonary vascular smooth muscle, PAFR protein expression and PAFR-mediated cell signaling may be inhibited via cross-talk between the cyclic nucleotides and PAFR-mediated responses. This hypothesis was investigated in ovine fetal pulmonary vascular smooth muscle cells using cAMP and cGMP on PAFR binding and PAFR-mediated cell signaling in both normoxia and hypoxia. After birth, during normoxia, pulmonary levels of cAMP rise significantly, perhaps contributing to the decrease in PAFR binding, PAFR protein expression and the ensuing fall in pulmonary vascular resistance. We employed siRNA to the catalytic domain of PKA to define the role of PKA in decreased pulmonary PAFR-mediated responses at birth.

## 2. Materials and methods

### 2.1. Materials

The study was approved by the Institutional Animal Care and Use Committee of Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center. Pregnant ewes (146-148 d gestation, term being 150 d) were purchased from Nebekar Farms (Santa Monica, CA). Authentic standards of PAF (C<sub>16</sub>-PAF) as well as 8-Br-cAMP, Rp-cAMPS, 8-Br-cGMP, Rp-8-pCPT-cGMPS were purchased from Biomol, Plymouth Meeting, PA. Radiolabeled PAF standards and substrates: hexadecyl-2-acetyl-sn-glycerol-3-phosphorylcholine, 1-O-[acetyl-<sup>3</sup>H-(N)]-, (<sup>3</sup>H-acetyl-C<sub>16</sub>-PAF), 21.5 Ci/mmol (370 GBq/mmol), and <sup>3</sup>H-thymidine were purchased from Perkin Elmer Life Sciences (Boston, MA). siRNA to PKA- $\alpha$  and its control were purchased from Cell Signaling Technologies (Carlsbad, CA). Phenylmethanesulfonyl fluoride, leupeptin, pepstatin, as well as bovine serum albumin, were purchased from Sigma Chemical Company (St. Louis, MO). Antibody to PKA and PKG were purchased from Cell Signaling, while PAFR antibody was purchased from Cayman Chemical Company (Ann Arbor, MI). Studies were done with freshly made reagents. Ecolite(+) liquid scintillation cocktail was purchased from MP Biochemicals (Irvine, CA).

### 2.2. Methods

Arterial intrapulmonary vessels were isolated from freshly killed term fetal lambs and then smooth muscle cells were harvested from the excised arteries and veins under sterile conditions as previously reported [20, 32]. Cells were used at the 3rd to 10th passage. Cell phenotype did not change from 1st to 10th passage as determined by the expression of  $\alpha$ -smooth muscle actin and myosin light-chain kinase proteins.

### 2.3. Study conditions

Studies were done with adherent cells in normoxia and in hypoxia.

*Normoxia:* Cells were studied in humidified incubator at 37 °C aerated with 5% CO<sub>2</sub> in air.

*Hypoxia:* An incubator set at 37 °C was first equilibrated for at least 1 h with a gas mixture of 2% O<sub>2</sub>, 10% CO<sub>2</sub>, and balance N<sub>2</sub> to maintain incubator culture media pO<sub>2</sub> < 40 Torr, and to

mimic fetal lung environment, and monitored with TED 60T percent oxygen sensor, Teledyne Analytical Instruments (City of Industry, CA). Cells were then placed in this equilibrated incubator and continuously aerated with the hypoxia gas mixture throughout the duration of the study.

## 2.4. Study of PAFR binding

### 2.4.1. General protocol

Receptor-binding assays were performed during hypoxia and normoxia as we previously reported [30]. Briefly, after incubation in normoxia or hypoxia, unbound  $^3\text{H}$ -PAF was washed off with ice-cold phosphate buffer saline, and then incubated on ice for 30 to 45 min in saline/EDTA mixture containing 154 mM saline and 5 mM EDTA. Receptor bound  $^3\text{H}$ -PAF was extracted on Whatman GF/C membrane filters using inline vacuum system. Then culture flasks or dishes were washed with calcium-free 0.25% bovine serum albumin-containing Tyrodes buffer, pH 6.4. Cell-bound PAF radioactivity was quantified by scintillation spectrometry (Beckman Instruments, Fullerton, CA). In studies probing the interaction of PAF with its receptors in the presence of other agonist or antagonists, cells were pre-incubated with the agent before the addition of  $^3\text{H}$ -PAF, and then incubated further according to the specific experimental protocol.

### 2.4.2. Specific protocols

*Effect of cAMP/cGMP on PAFR binding:* Cells were pre-incubated for 30 min, in normoxia or hypoxia, with buffer for controls or 10  $\mu\text{M}$  each of cell permeable cyclic nucleotide analogs, 8-Br-cAMP or 8-Br-cGMP; the cAMP/PKA receptor inhibitor Rp-cAMPS or the cGMP/PKG receptor inhibitor Rp-8-pCPT-cGMPS. Then 1.0nM  $^3\text{H}$ -PAF was added and incubated for 30 min more.

*Effect of cAMP/cGMP on PAFR protein expression:* Cells were pre-incubated for 3 hr min, in hypoxia or normoxia, with buffer for controls or 10  $\mu\text{M}$  each of cell-permeable cyclic nucleotide analogs, 8-Br-cAMP or 8-Br-cGMP. Membrane proteins were isolated from cultured cells and probed for PAFR protein expression by Western blotting.

*Proliferation assay:* Sub-confluent cells were serum starved by culturing in 0.1% fetal bovine serum (FBS) for 72 hr, then cells were cultured in 10% FBS with or without the test agents in the presence of 5 $\mu\text{Ci}$ /well of  $^3\text{H}$ -thymidine and incubated for 24 hr more in the oxygen condition [32]. Cell DNA that were labeled with  $^3\text{H}$ -thymidine were extracted with 0.5N NaOH and quantified on LKB 6500 scintillation counter (Beckman Coulter, Fullerton, CA).

*Transient cell transfection:* Cells were seeded in 6-well culture plates at  $5 \times 10^4$  cells per well in an antibiotic-free growth media and allowed to stabilize for 24 h. Then they were treated with 1.5 $\mu\text{g}/\text{ml}$  of each plasmid in lipofectamine transfection reagent with 50nM of PKA-C $\alpha$  siRNA according to the vendor's protocol (Cell Signaling) and incubated for 48 hr after which the transfection medium was replaced with fresh 10% FBS culture medium, which was also used to study cell proliferation or to study PAFR binding. Transfection efficiency was between 25%



and 35% within 24 hr of transfection as judged by the pGFP fluorescence [32]. The proliferative phenotype of transfected cells was compared to that of untransfected cells. In studying cell proliferation or PAFR binding, transfected cells were incubated for 24 hr for proliferation study or 30 min for PAFR binding, with and without 10nM PAF in 10% FBS. Control for cell proliferation is 10% FBS alone while cells transfected with scrambled siRNA (sham siRNA) were used as control for PKA-C $\alpha$  siRNA effect.

## 2.5. Western blotting

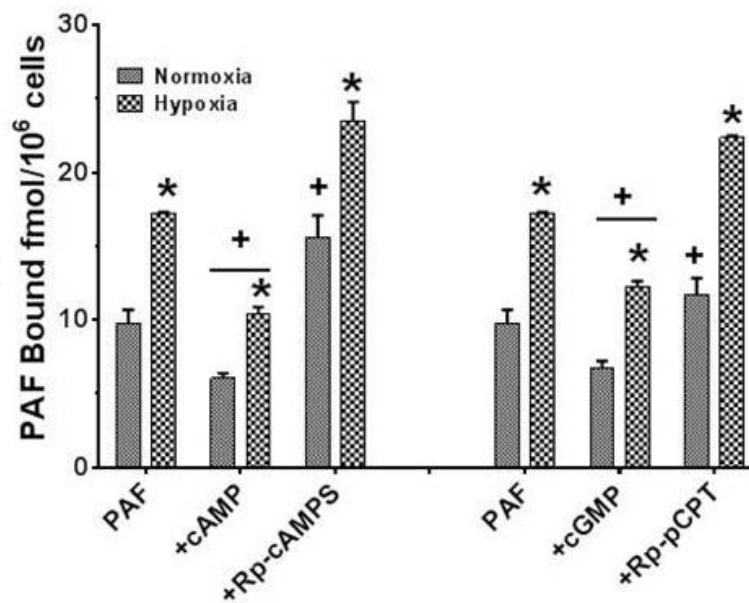
*Preparation of proteins for Western analysis:* Proteins were prepared from stimulated and unstimulated cells that were studied in normoxia or hypoxia according to our previous reports. Proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Western blotting for PAFR quantification following our published methods [20, 30, 32, 33]. After x-ray exposure, band corresponding to PAFR protein was quantified and normalized to  $\beta$ -actin density.

## 2.6. Data analysis

All numerical data are mean  $\pm$  SEM. In all instances where radioisotope was used, background radioactivity was subtracted before quantifying radioactivity. Data were analyzed with two-tailed *t*-test followed with ANOVA (GraphPad Prism 6, San Diego, CA). Results were considered significant at  $p < 0.05$ .

## 3. Results

Figure 1 shows the effect of 8-Br-cAMP and 8-Br-cGMP on PAF binding to its receptors in pulmonary arterial smooth muscle cells (PASMC) (fmol/10<sup>6</sup> cells). For both 8-Br-cAMP and 8-Br-cGMP, data are mean  $\pm$  SEM,  $n = 6$ ; \* $p < 0.05$ , different from normoxia; † $p < 0.05$ , different from PAF alone in normoxia or hypoxia. Cells treated with 10nM PAF alone, binding in normoxia was  $9.7 \pm 1.0$ , which increased to  $17.2 \pm 1.0$  in hypoxia. Pretreatment of cells with 10 $\mu$ M of 8-Br-cAMP decreased PAF by 31% in normoxia and by 29% in hypoxia, respectively. Inhibition of endogenous cAMP effect with 1 $\mu$ M of the cAMP/PKA receptor inhibitor, Rp-cAMPS in normoxia or hypoxia increased PAF binding by 65% compared to effect of PAF alone in normoxia and by 35% compared to effect of PAF alone in hypoxia. Also, pretreatment of cells with 10 $\mu$ M Br-cGMP decreased PAF binding by 38% and 40% in normoxia and hypoxia, respectively, compared to the effect of PAF alone. Inhibition of endogenous cGMP activity with the cGMP/PKG receptor antagonist Rp-8-pCPT-cGMPS (Rp-pCPT) increased PAF binding by 20% compared to PAF alone in normoxia, and by 30% in comparison to binding by PAF alone in hypoxia. In general, hypoxia increased PAFR binding; exogenous cAMP or cGMP attenuated PAFR binding while inhibition of endogenous cAMP or cGMP activity with specific cAMP/PKA or cGMP/PKG receptor antagonists restored PAFR binding to levels higher than effect of PAF alone.

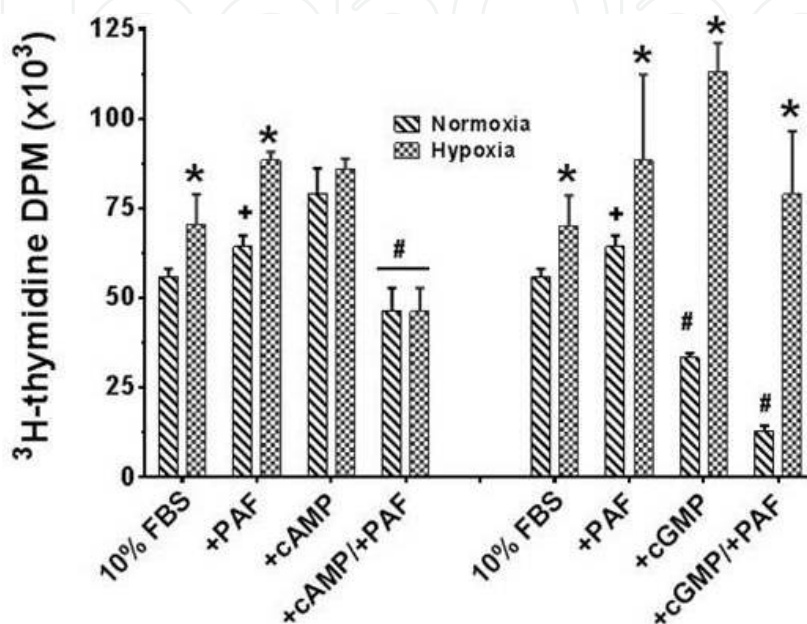


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**Figure 1.** Effect of cAMP and cGMP on PAF receptor binding FPASMC. Data are means  $\pm$  SEM,  $n = 6$ . Cells were pre-incubated for 30 min in normoxia or hypoxia with buffer alone, or  $10\mu\text{M}$  of 8-Br-cAMP or  $10\mu\text{M}$  of 8-Br-cGMP then  $10\text{nM}$  PAF was added and incubated for 30 min more as described in the methods section. Hypoxia increased PAF receptor binding and both 8-Br-cAMP and 8-Br-cGMP inhibited PAF receptor binding in normoxia and hypoxia. The specific inhibitors of PKA and PKG signaling; Rp-cAMPs and Rp-8-pCPT-cGMP reversed inhibitory effects of 8-Br-cAMP and 8-cGMP. \* $p < 0.05$ , different from PAF normoxia; + $p < 0.05$ , different from PAF alone in normoxia or hypoxia.

Effect of cyclic nucleotides cAMP and cGMP on PAF stimulation of fetal pulmonary arterial smooth muscle cells (FPASMC) growth ( $^3\text{H}$ -thymidine DPM  $\times 10^3$ ) is shown in Figure 2. For both 8-Br-cAMP and 8-Br-cGMP, data are mean  $\pm$  SEM,  $n = 6$ , \* $p < 0.05$ , different from normoxia; + $p < 0.05$ , different from PAF alone in normoxia; # $p < 0.05$ , different from PAF alone in normoxia or hypoxia. Treatment of cells with  $10\text{nM}$  of PAF in normoxia caused a 15% increase in cell proliferation compared to effect of FBS control ( $55.9 \pm 2.2$ ). In hypoxia, PAF increased cell proliferation by 25% compared to effect of FBS control in hypoxia ( $70.5 \pm 8.4$ ). Incubation with  $10\mu\text{M}$  8-Br-cAMP in normoxia or hypoxia produced no difference in cell proliferation compared to PAF effect in hypoxia (Figure 2a). However, co-incubation of cells with  $10\text{nM}$  PAF in the presence of cAMP significantly inhibited cell proliferation below effect of FBS control or  $10\text{nM}$  PAF in normoxia and hypoxia. With cGMP (Figure 2b), treatment of cells with  $10\mu\text{M}$  8-Br-cGMP in normoxia or hypoxia decreased cell proliferation by 40% in normoxia compared to FBS control, and by 48% in compared to effect of  $10\text{nM}$  PAF in normoxia. On the other hand, 8-Br-cGMP significantly increased cell proliferation compared to the effect of FBS control and  $10\text{nM}$  PAF in hypoxia. Co-incubation of cells with PAF and 8-Br-cGMP in normoxia decreased cell proliferation by 77% in compared to FBS control and by 80% compared to PAF alone. Co-incubation with 8-Br-cGMP and PAF in hypoxia produced no significant difference in cell growth compared to effects of FBS and  $10\text{nM}$  PAF in hypoxia. Thus, cAMP and cGMP produced different effects of PAF stimulation of FPASMC growth during normoxia and hypoxia.

Figure 3 shows representative Western blots of effect of cAMP and cGMP on PAFR protein expression. For both 8-Br-cAMP and 8-Br-cGMP, data are mean  $\pm$  SEM,  $n = 3$ . The statistics are as shown in the figures. The figures are not meant to be a cross-comparison of the effects of 8-Br-cAMP and 8-Br-cGMP on PAFR protein expression, but rather to compare effect of each cyclic nucleotide on PAFR protein expression. However, the figures show that hypoxia increased PAFR protein expression and in both cases, cAMP (Figure 3a) and cGMP (Figure 3b) decreased PAFR protein expression compared to control conditions.



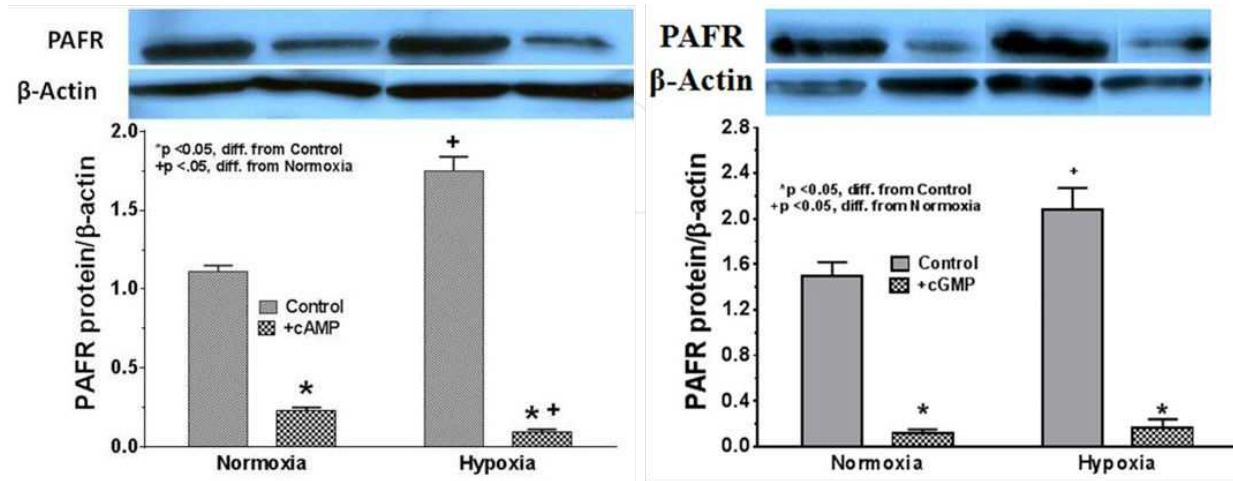
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**Figure 2.** Effect of cAMP and cAMP on PAF stimulation of FPASMC proliferation. Data are means  $\pm$  SEM,  $n = 6$ . Cells were pre-incubated for 30 min with buffer alone, or with 10  $\mu$ M 8-Br-cAMP, or 10 $\mu$ M of 8-Br-cGMP, then PAF was added and incubated for 24 hr more as described in methods section. PAF increased cell proliferation in normoxia and hypoxia. Effect of 8-Br-cAMP on cell proliferation in normoxia or hypoxia, co-incubation of 8-Br-cAMP and PAF decreased PAF-induced cell proliferation. 8-Br-cGMP alone decreased cell proliferation in normoxia, and co-incubation with PAF further decreased PAF stimulation of cell proliferation. \* $p < 0.05$ , different from normoxia; + $p < 0.05$ , different from effect of 10% FBS; # $p < 0.05$ , different from PAF effect.

We then investigated the effect of PKA siRNA PAFR binding and PAF stimulation of FPASMC proliferation in normoxia only. For both binding and proliferation, data are mean  $\pm$  SEM,  $n = 6$ ; \* $p < 0.05$ , different from 10% FBS control, + $p < 0.05$ , different from PAF alone, # $p < 0.05$ , different from 10% FBS control, PAF alone, and 8-Br-cAMP (+cAMP). In Figure 4a, 8-Br-cAMP decreased PAFR binding by 26% (PAF alone control, 10.2 fmol/10<sup>6</sup> cells). Pretreatment of cells with 50nM of the PKA-C $\alpha$  siRNA increased PAFR binding by 47% compared to effect of PAF alone (control) and by 52% compared to effect of cAMP alone. The effect of sham PKA-C $\alpha$  siRNA (sham) was not different from PAF alone. In Figure 4b, PAF alone stimulated greater cell proliferation (<sup>3</sup>H-thymidine DPM  $\times 10^3$ ) by 45% compared to 10% FBS control (101.2  $\pm$  5.3). Effect of 8-Br-cAMP on cell proliferation was 120.3  $\pm$  7.8 which is 19% lower than effect of 10% FBS control and 20% lower than effect of 10nM PAF alone. As with PAFR binding, PKA-C $\alpha$  increased cell proliferation and reversed the effect of cAMP on PAF binding. PAFR binding

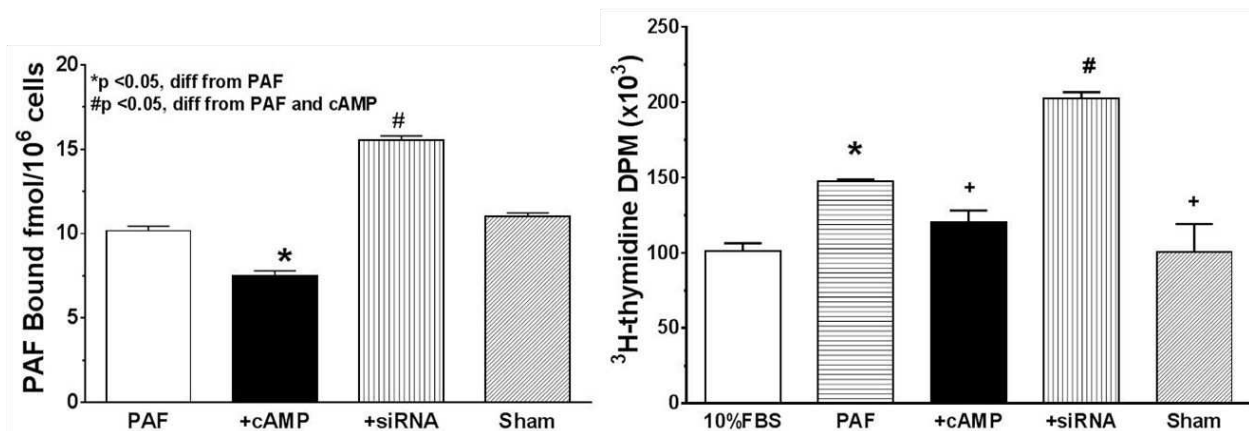


produced a 2-fold increase in cell proliferation compared to 10% FBS control and 37% increase compared to effect of 10nM PAF. The effect of the sham siRNA control was not different from effect of 10% FBS control.



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**Figure 3.** Effects of cAMP and cGMP on PAF receptor protein expression in normoxia and hypoxia. Data are means  $\pm$ SEM,  $n=3$ . Cells were incubated in for 24 hr in normoxia or hypoxia with buffer alone, or with 10 $\mu$ M of 8-Br-cAMP, figure 3a, or with 10 $\mu$ M 8-Br-cGMP, figure 3b. PAF receptor (PAFR) expression was measured by Western blotting and normalized to expression of beta-actin standard. Rp-8-pCPT-cGMPS, then 5nM PAF was added as needed and incubated for 20 min more. Treatment of cell with 8-Br-cAMP or 8-Br-cGMP suppressed PAFR protein expression in normoxia and hypoxia. \* $p < 0.05$ , different from control; + $p < 0.05$ , different from normoxia.



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**Figure 4.** Effects of cAMP and and siRNA to PKA- $\alpha$  on PAFR binding and PAF stimulation of cell proliferation. Data are means  $\pm$  SEM,  $n=5$ . Studies were done as described in methods section. PAFR binding (figure 4a) and cell proliferation were determined. PAFR binding was attenuated by 8-Br-cAMP, but siRNA to PKA- $\alpha$  increased PAFR binding. Effect of sham siRNA was not different from PAF alone. In figure 4b, 8-Br-cAMP decreased cell proliferation compared to effect of PAF alone. siRNA to PKA  $\alpha$  increased cell proliferation. Effect of sham siRNA was not different from 10% FBS or 8-br-cAMP. \* $p < 0.05$ , different from PAF alone of 10% FBS control; + $p < 0.05$ , different from PAF alone; # $p < 0.05$ , different from all other condition.

## 4. Discussion

In fetal pulmonary circulation, vasomotor tone is maintained high by multiple mechanisms such as elevated calcium, PAF, and thromboxane A<sub>2</sub> [22, 34, 35]. At birth, endogenous vasodilators induce smooth muscle relaxation via increased intracellular concentrations of the cyclic nucleotides, cAMP and cGMP, acting through their respective receptors PKA and PKG [27]. We have published previously that in ovine fetal pulmonary vascular smooth muscle cells, hypoxia upregulates PAFR binding and PAFR-mediated intracellular IP<sub>3</sub> and calcium release [30], suggesting that the hypoxic environment of the fetus facilitates PAFR binding and PAFR-mediated signaling and the maintenance of a high pulmonary vasomotor tone in utero. The possibility that vasoconstrictors such as PAF may actively downregulate vasodilator pathways in the hypoxic environment of fetal pulmonary circulation is being actively explored. Similarly, the decreased PAFR-mediated activity in the higher oxygen environment of the postnatal lung may involve the downregulation of PAFR-mediated cell signaling by other endogenous mediators such as cAMP and cGMP [36, 37]. The present report investigates the interaction between cyclic nucleotides, cAMP and cGMP, and PAF signaling pathway in FPASMC. We have found that in ovine fetal pulmonary vascular smooth muscle cells, both cAMP and cGMP decrease PAFR binding in normoxia through the actions of their respective kinases, PKA and PKG. Addition of PAF in physiologic concentrations to pulmonary venous smooth muscle cells decreased PKA and PKG protein expression and kinase activities during normoxia and hypoxia, suggesting that in the hypoxic environment of the fetal lungs, PAF may be actively downregulating cAMP- and cGMP-dependent signaling pathways and that postnatally, in the normoxic environment, cAMP and cGMP actively inhibit PAF binding to its receptors and PAFR-mediated signaling. This cross-talk between the two pathways will effectively maintain a high pulmonary vasomotor tone in utero and facilitate vasorelaxation at birth.

### 4.1. Cyclic nucleotides inhibit PAFR binding and PAFR-mediated cell proliferation

*Role of cAMP:* cAMP production is linked to  $\beta$ -adrenergic receptor-mediated activation of adenylyl cyclase [38, 39]. Inhibition of cAMP- and cGMP-dependent phosphodiesterases (PDEs) can also result in high cellular levels of cAMP and cGMP [38, 40], but the effect of cAMP and cGMP on PAFR-mediated signaling in PASMOC has not been reported. Unlike in pulmonary venous smooth muscle cells, the cell permeable analog of cAMP, 8-Br-cAMP, decreased PAFR binding in normoxia and hypoxia. Inhibition of cAMP-dependent PKA, the endogenous receptor of cAMP, with Rp-cAMPS, reversed the inhibitory effect of endogenous cAMP and significantly increased PAFR binding. Also, when PKA was inhibited with Rp-cAMPS, PAF binding in hypoxia was increased beyond the effect of addition of exogenous PAF alone. This suggests that cAMP interacts with PAFR after activation of its own receptor, PKA.

Effect of cAMP downstream from PAFR in the nucleus of PASMOC is not clear. 8-Br-cAMP enhanced cell growth in normoxia, with no change in cell growth in hypoxia compared to PAF effect in hypoxia. However, when cells were pulsed with 8-Br-cAMP and exposed to PAF, cell proliferation was significantly decreased in normoxia and hypoxia, suggesting that cAMP

effect occurs after activation of its receptor. This relationship is relevant physiologically because it suggests that postnatally, cAMP will stimulate growth of PASMC and under this condition, the presence of PAF will be detrimental cell growth and pulmonary vascular development. Thus, we can speculate that cAMP/PKA-mediated inhibition of PAF effects, *in vivo*, may constitute one mechanism whereby the postnatal vasodilator properties of cAMP are maintained. Our studies with PKA siRNA demonstrate that cAMP acts at its receptor, PKA, to inhibit postnatal adverse PAFR-mediated responses in the pulmonary circulation of the newborn lamb lung.

*Role of cGMP:* Endothelium-derived nitric oxide produced under basal conditions, or by a stimulus, readily diffuses into the contiguous smooth muscle to activate soluble guanylyl cyclase, resulting in an increase in cGMP synthesis and smooth muscle relaxation [37, 38]. Nitric oxide is a potent vasodilator in the pulmonary circulation and it is important in the transition of the pulmonary circulation from fetal to postnatal life. In this study, 8-Br-cGMP blunted PAFR binding to PASMC in physiologic oxygen level (normoxia) and in hypoxia. This suggests that cGMP produced *in vivo* will counteract the vasoconstricting properties of PAF. Interestingly, inhibition of endogenous cGMP activity with the inhibitor of cGMP-dependent PKG, the endogenous receptor of cGMP, resulted in abrogation of the ability of cGMP to inhibit PAF binding to its receptors, similar to the effect of PKA receptor antagonist. This shows that cGMP acts via its receptor to inhibit PAFR binding.

cGMP has been shown to inhibit endothelin-stimulated inositol phosphate release in pulmonary artery of fetal lambs studied in organ bath [41]. Inositol phosphate is released downstream from PAFR effect. Both endothelin and PAF are potent endogenous vasoconstrictors in the pulmonary circulation. This shows that increased levels of endogenous PAF under normoxic conditions can inhibit cGMP effect and as a corollary, increased levels of cGMP after birth can inhibit PAF effect in normoxia, leading to increased vasodilation. Our data show that cAMP and cGMP produce different effects on cell proliferation. During normoxia, cGMP inhibited PAF stimulation of cell proliferation, but the effect in hypoxia seemed to be stimulatory. This physiological significance of this effect is not clear, but may indicate a protective role of cGMP against unwarranted cell growth in the presence of PAF.

We can infer that *in vivo*, activation of PKA will result in inhibition of PAFR-mediated effects such as stimulation of inositol phosphate release, calcium mobilization, and vasoconstriction. As with cGMP effect, cAMP/PKA-mediated inhibition of PAF effects, *in vivo*, may constitute one mechanism whereby the postnatal vasodilator properties of cAMP are maintained.

#### **4.2. PAF and regulation of PKG and PKA activity and role in perinatal pulmonary adaptation**

Previous reports have shown that the activities of PKG and PKA are upregulated by normoxic condition [36, 37, 42]. The physiologic implications of these findings are that upregulation of PKG and PKA activities by normoxia, after birth, is one mechanism by which fetal high pulmonary vasomotor tone is downregulated to facilitate postnatal pulmonary adaptation. It can be deduced that favorable perinatal pulmonary vascular adaptation can be achieved by both downregulation of mediators of pulmonary vascular constriction, such as PAF, and upregulation of mediators of pulmonary vasodilation such as cGMP/PKG and cAMP/PKA.

Endothelin [43] and protein kinase C (PKC) [44] are two other mediators that have been reported to evoke vasoconstriction in the perinatal pulmonary circulation. In this report, we show that during normoxia, PAF significantly downregulates the activities of both cGMP- and cAMP-dependent protein kinases. We also found that exposure of the smooth muscle cells to 8-Br-cAMP and 8-Br-cGMP for 30 hr in normoxia and hypoxia, resulted in significant downregulation of PAFR in line with attenuation of PAFR binding to the cells. These findings strongly indicate that congenial perinatal pulmonary adaptation entails a combination of downregulation of PAFR-mediated effects by cyclic nucleotide-mediated pathways as we have shown in this report, as well as by upregulation of cGMP- and cAMP-mediated pathways as has been previously reported [31, 36, 37, 42].

PPHN is a pathological condition with different etiologies. High PAF levels have been reported in neonates with PPHN [31, 45], suggesting that persistence of high PAF levels postnatally may lead to abnormal perinatal pulmonary adaptation. In addition, we speculate that inhibition of PKG and PKA activities by high levels of PAF and the inability of the cyclic nucleotides to downregulate PAFR-mediated effects postnatally will also contribute to the development of PPHN.

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