Oxygen-dependent PAF receptor binding and intracellular signaling in ovine fetal pulmonary vascular smooth muscle

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Ibe, Basil O., Ada M. Portugal, Shiva Chaturvedi, and J. Usha Raj. Oxygen-dependent PAF receptor binding and intracellular signaling in ovine fetal pulmonary vascular smooth muscle. Am J Physiol Lung Cell Mol Physiol 288: L879–L886, 2005. First published December 23, 2004; doi:10.1152/ajplung.00341.2004.—Circulating levels of platelet-activating factor (PAF) are high in the fetus, and PAF is active in maintaining high PVR in fetal hypoxia (Ibe BO, Hibler S, Raj J. J Appl Physiol 85: 1079–1085, 1998). PAF synthesis by fetal pulmonary vascular smooth muscle cells (PVSMC) is high in hypoxia, but how oxygen tension affects PAF receptor (PAF-r) binding in PVSMC is not known. We studied the effect of oxygen tension on PAF-r binding and signaling in fetal PVSMC. PAF binding was saturable. PAF-r density (Bmax, fmol/106 cells; means ± SE, n = 6), 25.2 ± 0.77 during hypoxia (PO2 < 40 Torr), was higher than 13.9 ± 0.44 during normoxia (PO2 = 100 Torr). Kd was twofold lower in hypoxia than in normoxia. PAF-r protein expression, 35–40% greater in hypoxia, was inhibited by cycloheximide, a protein synthesis inhibitor, suggesting translational regulation. IP3 release, an index of PAF-r-mediated cell signaling, was greater in hypoxia (EC50: hypoxia, 2.94 ± 0.61; normoxia, 5.85 ± 0.51 nM). Exogenous PAF induced 50–90% greater intracellular calcium flux in cells during hypoxia, indicating hypoxia augments PAF-r-mediated cell signaling. PAF-r phosphorylation, with or without 5 nM PAF, was 40% greater in hypoxia. These data show J hypoxia upregulates PAF-r binding, PAF-r phosphorylation, and PAF-r-mediated intracellular signaling, evidenced by augmented IP3 production and intracellular Ca2+ flux; and 2 hypoxia-induced PAF-r phosphorylation results in activation of PAF-r-mediated signal transduction. The data suggest the fetal hypoxic environment facilitates PAF-r binding and signaling, thereby promoting PAF-mediated pulmonary vasoconstriction and maintenance of high PVR in utero.

PAF-evokes its effects by binding to its G protein-coupled receptor (GPCR), which is a seven-transmembrane receptor (9, 47). Ligand binding to GPCR results in the regulation of signal transduction pathways, by processes involving activation, desensitization, resensitization, and inactivation (18, 40). In this report, our main objective was to investigate the mechanisms by which oxygen tension modulates PAF receptor binding and PAF-mediated intracellular signaling in fetal pulmonary vascular smooth muscle cells (PVSMC). Because the fetus is normally exposed to a low oxygen tension, we were particularly interested in studying the effects of a low oxygen tension on PAF-receptor interactions. We used cultured vascular smooth muscle cells from intrapulmonary vessels of fetal lambs and exposed them to different oxygen tensions. We determined the effect of hypoxia on PAF receptor protein expression, measured PAF-receptor binding, PAF receptor phosphorylation, as well as inositol triphosphate (IP3) and intracellular Ca2+ release as indexes of PAF receptor-mediated intracellular signaling.

MATERIALS AND METHODS

Materials

Pregnant ewes (146–148 days gestation, term being 150 days) were purchased from Nebekar Farms (Santa Monica, CA). Authentic standards of PAF, hexadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (C16-PAF), hexadecyl-sn-glyceryl-3-phosphorylcholine (Lyso-C16-PAF), octadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (C18-PAF) as well as okadaic acid, calyculin, leupeptin, PMA, and genistein were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Fura-2 pentakis (acetoxymethyl) ester (Fura-2 AM) was purchased from Sigma (St. Louis, MO). Radiolabeled PAF standards and substrates were purchased from Perkin Elmer Life Sciences (Boston, MA). They are: myo-[2-3H(N)]-inositol, hexadecyl-2-acetyl-sn-glyc-

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erythritol, 1-O-[acetyl-3H(N)], ([3H]acetyl-C6-PAF), specific activity 13.5 Ci/nmol (370 GBq/nmol). Antibody to PAF receptor was purchased from Cayman Chemical (Ann Arbor, MI). Ecolite(+) liquid scintillation cocktail was purchased from ICN Biochemicals (Irvine, CA).

Methods

Study of PAF receptors in ovine fetal PVSMC. PREPARATION OF SMOOTH MUSCLE CELLS. Intrapulmonary vessels (2nd–4th generation) were isolated from term fetal lambs, and then smooth muscle cells (SMC) were harvested under sterile conditions as previously reported (29, 48). Cells were used at the 4th–6th passage. Identity of SMC was determined with an SMC-specific monoclonal antibody (Sigma), and it was ascertained that SMC cultures were devoid of endothelial cells and fibroblasts. Cell phenotype did not change from 4th to 6th passages as determined by the expression α-smooth muscle actin and myosin light chain kinase proteins.

STUDIES WITH SMC. In this report, all experiments were done in PVSMC harvested from intrapulmonary veins, as they exhibit more PAF binding than cells from arteries. We have also previously reported that in the fetus, pulmonary veins demonstrate significant vasoactivity, at times greater than that of pulmonary arteries, and contribute a significant fraction to total vascular resistance (33, 49, 54).

Experimental conditions. NORMOXIA. Cells were studied in a humidified incubator at 37°C aerated with 5% CO2 in air. PO2 in cell media was maintained at 100 Torr, measured with a Nova Stat Profile 3 blood-gas instrument (Nova Biomedical, Waltham, MA) (29).

HYPOXIA. For hypoxia, the incubator was first equilibrated with a gas mixture of 2% O2, 10% CO2, balance N2 to maintain PO2 contribution a significant fraction to total vascular resistance (33, 49, 54).

Studies of PAF receptor binding. ASSAY STANDARDIZATION. Cells were incubated with 1.0 nM [3H]acetyl-C6-PAF ([3H]PAF) for 30 min at 37°C as previously reported (36). Total PAF receptor binding (fmol/106 cells, n = 6) in cells by 30 min during normoxia was 28.70 ± 2.71 compared with 43.48 ± 0.71 during hypoxia. Optimum PAF receptor binding conditions were determined by incubating PVSMC in normoxia with 1.0 nM of [3H]PAF with or without 500 nM of nonradiolabeled PAF at 4°C for 24 h or at 20°C and 37°C for 30 min. There was no difference in PAF binding after incubation of cells at 20°C and 37°C for 30 min (specific binding was 10.11 ± 0.32); however, binding was lower after incubation at 4°C for 24 h (specific binding of 3.32 ± 0.14). Binding assay was further standardized with respect to duration of incubation (0–120 min), buffer pH, and BSA content. From the results of these standardization assays, the rest of the experiments were done with PVSMC at 37°C with Dulbecco’s modified Eagle’s medium (DMEM), pH 7.4, containing 1% BSA.

GENERAL ASSAY PROTOCOL. Cells were washed with phosphate-buffered saline (PBS) before use. After incubation in normoxia or hypoxia, unbound [3H]PAF was washed off with ice-cold PBS. Then a mixture of 154 mM saline and 5 mM EDTA (36) was added to the cells and incubated on ice for 30–45 min. [3H]PAF bound to its receptor was extracted from cells on Whatman GF/C membrane filters with a Whatman filter manifold and in-line vacuum system. Then, the culture flask or dish was washed with calcium-free Tyrode’s buffer, pH 6.4 (36), containing (in mM) 137 NaCl, 2.7 KCl, 11.9 NaHCO3, 1.0 MgCl2, 0.41 NaH2PO4, 5.0 HEPES, 5.6 glucose, and 0.25% BSA and transferred to the filtration manifold for extraction. Cell-bound PAF radioactivity was quantified by scintillation spectrometry (Beckman Instruments, Fullerton, CA). To study PAF interaction with its receptors in the presence of other agonist or antagonists, we preincubated cells with the agent or with buffer for control, then [3H]PAF was added and incubated further according to the specific experimental protocol.

Specific protocols. SPECIFICITY OF PAF RECEPTOR BINDING IN PVSMC. Studies were done as follows: 1) Saturation binding. Cells were treated with different concentrations of [3H]PAF (0–16 nM) and incubated for 30 min during normoxia or hypoxia with or without 500 nM nonradiolabeled PAF. 2) Effect of cycloheximide on PAF receptor protein expression. Cells were incubated in normoxia for 60 min with or without 30 μM cycloheximide, a protein synthesis inhibitor. 3) Effect of receptor antagonists on PAF binding. Cells were preincubated for 30 min with the PAF receptor antagonists CV-6209 (1.0 μM), WEB-2170 (1.0 μM), or the thromboxane A2 (TXA2) receptor antagonist SQ-29548 (30 μM) and then 1.0 nM of [3H]PAF was added, and the cells were incubated for 30 min more during normoxia or hypoxia.

Study of PAF-stimulated IP3 release. LABELING OF CELLS WITH [3H]MYO-INOSITOL. Cells were prelabeled with 10 μCi/ml of [3H]myo-inositol and incubated for 16–24 h in 5% CO2 in air, and studies were conducted as previously reported (3). We found that 16–24 h in culture is sufficient for optimum incorporation of inositol isotope (25%) into cells, and there was no difference in incorporation of inositol radioactivity in hypoxia or normoxia.

STIMULATION OF [3H]INOSITOL PHOSPHATE RELEASE. Each test stimulus was prepared in 10 mM LiCl buffer, made fresh on the day of experiment (3, 57). Labeled cells were stimulated with PAF or other agents and incubated in hypoxia or normoxia for 20 min at 37°C. We quenched reactions by adding 10 mM formic acid, cell suspension was loaded on preequilibrated AG 1-X8 columns, and [3H]inositol phosphates (IP3) were extracted as previously described (57). [3H]IP3 radioactivity was quantified by scintillation spectrometry.

Specific protocols. EFFECT OF PAF AND PMA ON IP3 RELEASE BY PVSMC. Cells prelabeled with [3H]myo-inositol were stimulated with 5 nM PAF or 10 nM PMA and incubated for 20 min in hypoxia or normoxia at 37°C.

EFFECT OF SPECIFIC INHIBITORS ON PAF-STIMULATED IP3 RELEASE. Cells prelabeled with [3H]myo-inositol were preincubated for 20 min at 37°C in normoxia with the specific inhibitor or with buffer for controls. Then 5 nM of PAF was added and incubated for 20 min more.

EFFECT OF EXOGENOUS PAF ON IP3 RELEASE. Cells prelabeled with [3H]myo-inositol were treated with buffer alone for baseline or stimulated with different concentrations (10–11–10–6 M) PAF and incubated for 20 min in normoxia or hypoxia at 37°C. [3H]IP3 released in each protocol was assayed as described above.

MEASUREMENT OF CA2+ RELEASE. Intracellular calcium flux was measured as previously reported (16, 36). In brief, cells in 10% FBS culture media were incubated for 1 h in hypoxia or normoxia without any stimulus and then washed with PBS. Washed cells were trypsinized, spun at 200 g for 5 min, and then washed with Ca2+-free modified Tyrode’s buffer containing (in mM) 140 NaCl, 2.7 KCl, 12 NaHCO3, 0.49 MgCl2, 0.37 NaHPO4, 25 HEPES, and 5.6 glucose. When required, CaCl2 was added to a final concentration of 1.0 mM (16). Cells were incubated for 70 min at 37°C in the calcium-free Tyrode’s buffer containing 5 μM of fura-2 AM. The buffer used on cells for hypoxia study was continuously aerated with the hypoxia gas mixture while buffer for normoxia study was aerated with a normoxia gas mixture containing 21% oxygen. After 70-min incubation in the 5 μM fura-2 AM solution, the tubes were spun to pellet the cells, which were then resuspended in Tyrode’s buffer containing 1.0 mM of CaCl2 and 0.1% BSA aerated with the hypoxia or normoxia gas mixtures. Then the cells resuspended in the 0.1% BSA calcium containing Tyrode’s buffer were dispensed in aliquots of 50,000 cells/tube into borosilicate assay tubes, and calcium/FURA-2 AM fluorescence was determined at 340/380 nm excitation and 510 nm emission (Hewlett Packard) after treatment with different concentrations of PAF. The following controls were used: 1) cell-free Ca2+-.
containing buffer aerated with normoxia or hypoxia gas mixtures; 2) Ca\(^{2+}\)-containing buffer with 50,000 cells/tube, aerated with the hypoxia or normoxia gas, but without PAF treatment.

**STUDY OF PAF RECEPTOR PHOSPHORYLATION.** Cells were pulsed for 90 min in normoxia with 150 μCi/dish of \(^{32}\)Porthophosphate in phosphate-free DMEM, to label intracellular pool of ATP, and then prepared for study as previously reported (3). Labeled cells were then incubated for 5 min in hypoxia or normoxia with buffer (baseline) or with 5 nM PAF. The cells were immediately placed in ice bath to quench the phosphorylation of receptors and then lysed with a hypotonic lysis buffer (3). Phosphorylated receptors were immunoprecipitated with anti-PAF receptor antibody, subjected to SDS gel electrophoresis with 20 μl of each immunoprecipitate, after which phosphorimaging analysis and densitometry were done on the immunoblots.

**PREPARATION OF PROTEINS FOR IMMUNOPRECIPITATION OF PHOSPHORYLATED PAF RECEPTOR.** All procedures were done at 4°C. Cells washed with phosphate-free buffer were lysed with 50 mM Tris lysis buffer, pH 8.0, containing 157 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 5 mM EDTA, 10 mM Na pyrophosphate, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aproptinin, 100 μg/ml 1-chloro-3-[4-tosylamido]-4-pheny-2-butanone, 50 μg/ml l-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone, and 10 μg/ml phenylmethylsulfonyl fluoride (PMSF) (3). Lysates were transferred to an Eppendorf tube and incubated for 90 min on a rotating platform followed with centrifugation at 5,000 g for 5 min. The supernatant was triturated for 1 h with 0.2% protein G-agarose in dialyzed BSA and dissolved in lysis buffer and recentrifuged as above. The resulting supernatant was treated with 10 μg of anti-PAF receptor antibody and mixed thoroughly for 1 h in an inversion platform followed by centrifugation. Supernatant was recovered, and phosphorylated PAF receptor was immunoprecipitated with 100 mM ammonium sulfate [(NH₄)₂SO₄], followed with a series of centrifugations and washes to pellet and purify the phosphorylated PAF receptor. Then purified pellet of phosphorylated PAF-R was suspended in 100 μl of 1X SDS-PAGE sample buffer, incubated for 10 min at 85–90°C, and then used for Western blotting on 15% SDS-polyacrylamide gel electrophoresis.

**Western blotting for PAF receptors in PVSMC.** PREPARATION OF PROTEINS FOR WESTERN BLOTTING. Briefly, at the end of the experiments, proteins were prepared from PVSMC with a 40 mM HEPES lysis buffer, pH 7.4 (1, 3), containing the following: 1 mM EGTA, 4 mM EDTA, 2 mM MgCl₂, 10 mM KCl, 1 mM DTT, 0.1 mM PMSF, 5 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μM 4-(2-aminoethyl) benzenesulfonyl fluoride, 200 mM sodium fluoride, 20 mM sodium pyrophosphate, 0.2 mM sodium vanadate, and 0.1 mg/ml trypsin inhibitor. Proteins were prepared from control and unstimulated cells and subjected to Coomassie blue analysis before use in Western blotting.

**SDS-PAGE ELECTROPHORESIS.** Proteins were suspended in SDS sample buffer, pH 6.8, containing 125 mM Tris base, 4% SDS, 0.006% bromphenol blue, 36 mM EDTA, 90 mM DTT, 10% glycerol, and 10% 2-mercaptoethanol. Samples were electrophoresed for 1 h at 200V on 4–12% Tris-glycine gradient gels (BioWhittaker), along with Bio-Rad kaleidoscope prestained molecular weight markers and protein standards. Studies were done to determine optimum conditions for electrophoresis of PAF receptor protein. After 1 h of SDS-PAGE, proteins were transferred to nitrocellulose membrane by means of mini Trans-Blot, (Bio-Rad) at 70 V for 35 min. Blots were blocked with 5% nonfat dry milk in 1% Tween 20-Tris-buffered saline (1% T-TBS) overnight. Blots were then incubated for 1 h with 1:500 dilution human PAF receptor antibody (monoclonal, Cayman), washed with 1% T-TBS, and incubated for 1 h with an anti-rabbit Ig horseradish peroxidase-linked secondary antibody (Amersham), followed by washes with 1% T-TBS. The signals were developed for 1 min with the Amersham ECL Western Blot detection kit and then exposed to X-ray film. Band corresponding to PAF receptor protein was scanned with EagleEye densitometer (Stratagene) to quantify blot density. With immunoprecipitated PAF receptor, after SDS-PAGE, the amount of phosphorylated PAF receptor was also quantified by phosphorimaging of phosphorylated receptor bands and reported as \(^{32}\)P radioactivity.

**DATA ANALYSIS.** All numerical data are means ± SE. In all instances where radioisotope was used, we subtracted background radioactivity before quantifying radioactivity. Data were analyzed with two-tailed t-test followed with ANOVA (Prism program). Results were considered as significant at \(P < 0.05\)

**RESULTS**

Figure 1 shows specific PAF binding and PAF receptor protein expression in PVSMC during normoxia and hypoxia. In Fig. 1A, binding in hypoxia was 40% greater than binding in

![Figure 1](https://example.com/figure1.png)
normoxia. Figure 1A shows a representative Scatchard analysis of PAF receptor density, which was higher in hypoxia than in normoxia. The dissociation constant ($K_d$) of PAF binding during hypoxia was 0.8 ± 0.1 and was twofold lower than $K_d$, 1.7 ± 0.5, in normoxia. PAF receptor protein expression was more in hypoxia than normoxia. Cycloheximide, a protein translation inhibitor, inhibited PAF receptor protein expression during normoxia and hypoxia (Fig. 1B).

Figure 2 illustrates the specificity of PAF binding to its receptors. The selective PAF receptor antagonist CV-6209 and WEB-2170, a competitive PAF receptor antagonist, both produced significant inhibition of PAF receptor binding during normoxia and hypoxia. However, the selective TxA2 receptor antagonist SQ-29548 was ineffective in inhibiting PAF receptor binding during normoxia. PAF binding to its receptor during hypoxia was augmented by 30% in the presence of SQ-29548, suggesting that TxA2 when bound to its receptor may somehow interfere with activation of the PAF receptor, so that the blocking of the TxA2 receptor results in augmentation of PAF receptor binding.

Figure 3 shows the effect of PMA and PAF on inositol phosphate (IP3) release (dpm/10^6 cells) during normoxia and hypoxia. During normoxia (Fig. 3A), baseline IP3 release was 896 ± 38. Treatment with PMA increased IP3 release fivefold, whereas with PAF treatment, IP3 release was increased 10-fold over baseline conditions and twofold higher than PMA-stimulated IP3 release. During hypoxia, baseline IP3 release was 1,790 ± 130. Treatment with PMA significantly increased IP3 release more than fourfold over baseline conditions. Treatment with PAF also increased IP3 release ninefold over baseline conditions and twofold over PMA effect. PAF-stimulated cells released more IP3 than cells stimulated with PMA. Hypoxia upregulated IP3 release under all the conditions studied.

Figure 3B shows the effect of specific inhibitors on PAF-stimulated IP3 release. The selective PAF receptor antagonist CV-6209 inhibited inositol phosphate release. Genistein, a tyrosine kinase inhibitor, had some nonspecific effect on IP3 release. But importantly, genistein inhibited PAF-stimulated inositol phosphate release, indicating that tyrosine kinase is involved in PAF-induced IP3 release. Figure 3C shows the dose-related effect of added PAF on IP3 release during hypoxia and normoxia. Release of IP3 by the cells was PAF concentration-dependent; IP3 release increased as PAF concentration was increased from 0.01 to 10 nM. At the highest concentration of PAF used, 1,000 nM, the amount of IP3 released decreased. This may be due to some toxic effects of this very high nonphysiological level of PAF. The EC50 (nM) of PAF-stim-
ulated IP₃ release during hypoxia was 2.94 ± 0.61, which was different from 5.84 ± 0.51, calculated during normoxia.

Figure 4 shows the effect of hypoxia on PAF-stimulated intracellular calcium flux. Release of Ca²⁺ from intracellular calcium stores was PAF concentration related, and at all concentrations of PAF studied, hypoxia augmented PAF-stimulated Ca²⁺ release. For instance, with 0.1 nM PAF, calcium release was 108 ± 4% of control during normoxia and 166 ± 5% during hypoxia, a 54% greater release than in normoxia. With 10 nM PAF, the values are 145 ± 2% during normoxia and 223 ± 4% during hypoxia. PAF-stimulated Ca²⁺ release attained a maximum (348 ± 19%) during hypoxia with 1,000 nM but not during normoxia (201 ± 6%). With 10,000 nM PAF, Ca²⁺ release during hypoxia dropped significantly to 312 ± 9%, whereas release during normoxia increased significantly to 256 ± 4%, but still lower than release during hypoxia. This decrease in Ca²⁺ release during hypoxia at the highest concentration of PAF used may be due to faster depletion of calcium stores during hypoxia.

Figure 5 shows PAF receptor phosphorylation during normoxia and hypoxia. Figure 5A shows the immunoblot of the phosphorylated receptors. Figure 5B shows the phosphorimage analysis of the ³²P radioactivity measured from immunoblots of the immunoprecipitated PAF receptors. During normoxia, baseline blot radioactivity was 4,148 ± 79. Stimulation with 5 nM PAF led to a 50% increase in radioactivity. During hypoxia, baseline ³²P radioactivity was 6,407 ± 1,084, which increased by >100% on stimulation with 5 nM PAF for 5 min. Baseline ³²P radioactivity during hypoxia was 36% higher than that during normoxia, and on stimulation with PAF, radioactivity during hypoxia was 40% higher than during normoxia. Figure 5C shows the densitometry of immunoblots of the phosphorylated PAF receptors for baseline studies and on stimulation with 5 nM PAF for 5 min. The densitometry data paralleled the results of phosphorimage analysis. In all instances, i.e., immunoblotting, phosphorimaging, and densitometry, the blot intensity was significantly greater in hypoxia.

DISCUSSION

Pulmonary vascular resistance in utero is high such that pulmonary blood flow is only 8–10% of total cardiac output (24). There are multiple mechanisms by which vasomotor tone is maintained high in the fetal pulmonary circulation. We have shown that, in fetal PVSMC, PAF synthesis is high (27) and that circulating levels of PAF are also high (25). We have also shown that endogenous PAF produced in the hypoxic fetal environment contributes to maintenance of the high pulmonary vasomotor tone in utero and if the action of PAF is blocked with a PAF receptor blocker, pulmonary vascular resistance falls dramatically (25). It has been shown that PAF induces cell proliferation via a PAF receptor-mediated mechanism and that
PAF-mediated cell proliferation occurs in different types of cells, including lung cells (15, 50). Furthermore, it has been reported that hypoxia induces proliferation of PVSMC (56). Hyperplasia of the pulmonary artery SMC is part of the pathophysiology of chronic hypoxia induced pulmonary hypertension and is mediated by various cytokines and immune modulators (38, 41, 50). Abnormal states of hypoxia in utero may therefore result in persistently high vasomotor tone as well as vascular remodeling and, in conjunction, with a high PAF level may lead to PPHN (8, 21).

In this study, we have investigated the effect of oxygen tension on PAF interactions with its receptor and on PAF-mediated intracellular signaling in ovine fetal PVSMC. We have demonstrated the presence of functional PAF receptors in these SMC because we found that the specific PAF receptor antagonists CV-6209 and WEB-2170 inhibited PAF binding to its receptors, whereas the TxA2 receptor antagonist SQ-29548 did not inhibit PAF binding, but rather SQ-29548 augmented PAF receptor binding during hypoxia. This suggests the existence of some cross talk between PAF and thromboxane in the SMC, as has been observed in isolated perfused rat lungs (39). Employing IP3 release and intracellular calcium flux by stimulated cells as indexes of PAF-receptor mediated cell signaling, we have demonstrated, for the first time, that hypoxia upregulates PAF receptor-mediated cell signaling in fetal PVSMC. This was accompanied by an upregulation by hypoxia of PAF receptor protein expression. Phosphorylation of PAF receptors occurred both during normoxia and hypoxia, and we have demonstrated that hypoxia significantly augmented PAF receptor phosphorylation. These results suggest that phosphorylation of PAF receptors in pulmonary vascular smooth muscle is an activation of PAF receptor-mediated cell signaling.

**PAF Receptor Binding and Receptor Protein Expression in PVSMC**

PAF receptor is a member of GPCR, a family of proteins with seven transmembrane loops (9, 31, 44, 47). Hypoxia upregulated PAF binding with 40% more receptors in hypoxia with \( K_d \) of 0.8 ± 0.1 in hypoxia, which was twofold lower than 1.7 ± 0.5 in normoxia, suggesting that during hypoxia, PAF binds more tightly to its receptors to initiate and sustain pulmonary vasoconstriction (25). Our finding that cycloheximide inhibited PAF receptor protein expression suggests a translational regulation of receptor expression during hypoxia. In endothelial cells and eosinophils, \( K_d \) of PAF binding was 0.043 and 2.3 nM, respectively (35, 36), whereas a \( K_d \) of 1.60 nM was reported for Chinese hamster ovary cells (16). Therefore the values of \( K_d \) in the PVSMC we have studied are within the values previously reported in other cell types.

**PAF Receptor Phosphorylation and Cell Signaling**

Phosphorylation or dephosphorylation is one mechanism of control of receptor-mediated cellular events (18, 22, 42). In this study, we found that phosphorylation of PAF receptors was greater in hypoxia. Quiescent GPCR characteristically maintain a heterotrimeric guanine nucleotide binding protein, GDP, designated as Go\( \beta \gamma \), and on activation, GPCRs undergo specific conformational changes (23), which ultimately result in initiation, attenuation, or cessation of cellular processes (14, 45). Phosphorylation of G proteins also promotes activity of some enzymes involved in intracellular cell signaling, including phospholipase C and phosphatidylinositol 3-kinase (2, 3, 9, 10, 53). As a GPCR, PAF receptor may be involved in all the molecular mechanisms of GPCR signaling involving activation, desensitization, resensitization, and inactivation. PAF receptor binding activates phosphoinositide phospholipase C, with release of IP3 and subsequent intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) mobilization (2, 9, 30). Our data show that PAF-stimulated IP3 release was concentration dependent as previously reported (2, 9, 30) and was augmented by conditions of hypoxia. The EC50 of PAF-stimulated IP3 production in hypoxia was ~10-fold lower compared with normoxia, suggesting increased PAF-receptor interactions and also more receptor sensitivity in hypoxia. When we tested for specificity, PAF-stimulated IP3 production was significantly inhibited by CV-6209, a selective PAF receptor antagonist, and by genistein, a protein tyrosine kinase inhibitor, indicating that IP3 release occurred by specific PAF receptor-mediated mechanisms via a GPCR pathway.

PAF binding to its receptors results in [Ca\(^{2+}\)]\(_i\) mobilization (11, 32, 36, 37). The effect of hypoxia on intracellular Ca\(^{2+}\) flux in ovine intrapulmonary venous SMC has not been described. In this study, hypoxia significantly increased PAF-stimulated Ca\(^{2+}\) release from intracellular stores by 50% at lower concentrations of PAF and by 90% at higher concentrations. This is in agreement with published report on bovine cerebral microvascular endothelial cells studied in normoxia, in which PAF-stimulated Ca\(^{2+}\) release ranged from ~5% at 1 nM to 145% at a 10,000 nM concentration of PAF (37). Also PAF and endothelin (ET)-1 have been reported to increase [Ca\(^{2+}\)]\(_i\) in endothelium and vascular SMC (6), and a recent report (13) showed that PAF and ET-1 increased intracellular calcium flux in rat mesenteric artery and vein. Hypoxia, together with an exogenous stimulus, led to an increase in intracellular calcium flux (5, 34, 43, 55). For instance, in rabbit pulmonary artery SMC, carbonyl cyanide m-chlorophenyl hydrazone, a mitochondrial protonophore, augmented [Ca\(^{2+}\)]\(_i\) increase in response to hypoxia (34). The mechanism of hypoxia-induced increase in intracellular calcium flux is not yet clear. Our data suggest that increased PAF receptor binding as well as increased inositol phosphate release may be one mechanism. Other reports have implicated involvement of sarcoplasmic reticulum calcium ATPase (20) and R-type calcium channels (7), among others. We can also speculate on the involvement of calcium/calmodulin (CaM) kinases in the regulation PAF-stimulated calcium release in these cells. Studies have shown that CaM kinases exert significant regulatory effects on responses of some vascular SMC to specific stimuli (19, 42, 52, 58). Furthermore, our data show that hypoxia-induced increase in PAF receptor binding is linked with increased receptor phosphorylation and increase in IP3 release (an index of PAF-mediated cell signaling) and is coupled to increased intracellular calcium flux. These series of links suggest that PAF receptor phosphorylation in ovine fetal PVSMC is an activation of PAF receptor-mediated cell signaling rather than an inactivation or desensitization process. Our data also suggest that in the hypoxic environment of fetal lungs, increased release of IP3 by PAF independently or in conjunction with a relevant endogenous ligand such as endothelin will lead to increased intracellular calcium levels in the

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cells, resulting in greater SMC contraction and maintenance of a high pulmonary vasomotor tone in utero.

**REFERENCES**


