Published in final edited form as:

Mol Genet Metab. 2010 December ; 101(4): 400–408. doi:10.1016/j.ymgme.2010.08.005.

Prolonged hypoxia modulates platelet activating factor receptor-mediated responses by fetal ovine pulmonary vascular smooth muscle cells

Lisette S. Renteria¹, J. Usha Raj², and Basil O. Ibe¹

¹ Department of Pediatrics, David Geffen School of Medicine at UCLA, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, CA 90502

² Department of Pediatrics, University of Illinois at Chicago, Chicago, IL 60612

Abstract

Hypoxia augments PAF receptor (PAFr) binding and PAFr protein expression in venous SMC (SMC-PV). We compared effect of acute and prolonged hypoxia ($pO_2 < 40$ torr) on PAFr-mediated responses in arterial SMC (SMC-PA) and SMC-PV. Cells were studied for 30 min (acute) or for 48h (prolonged) hypoxia and compared to normoxic ($pO_2 \sim 100$ torr) conditions. PAF binding was quantified in fmol/ 10^6 cells (means $\pm$ SEM). PAF binding in normoxia were: SMC-PA, 5.2 ± 0.2 ; in SMC-PV, 19.3 ± 1.1 ; values in acute hypoxia were: SMC-PA, 7.7 ± 0.4 and in SMC-PV, 27.8 ± 1.7 . Prolonged hypoxia produced 6-fold increase in binding in SMC-PA, but only 2-fold increase in SMC-PV, but binding in SMC-PV was still higher. Acute hypoxia augmented inositol phosphate release by 50% and 40% in SMC-PA and SMC-PV respectively. During normoxia, PAFr mRNA expression by both cell types was similar, but expression in hypoxia by SMC-PA was greater. In SMC-PA, hypoxia and PAF augmented intracellular calcium flux. Re-exposure of cells to 30 min normoxia after 48h hypoxia decreased binding by 45–60%, suggesting immediate down-regulation of hypoxia-induced PAFr-mediated effects. We speculate that re-oxygenation immediately reverses hypoxia effect probably due to oxygen tension-dependent reversibility of PAFr activation and suggest that exposure of the neonate to prolonged state of hypoxia will vilify oxygen exchange capacity of the neonatal lungs.

Keywords

smooth muscle cells; arteries; veins; PAF receptor mRNA; calcium flux

Introduction

Platelet activating factor, 1-O-alkyl-2-acetyl-sn-glycerol-3-phosphorylcholine (PAF), is a phospholipid with a wide range of biological activities [1,2], including action as a potent vasoconstrictor in the pulmonary circulation [3–6]. In fetal lambs, endogenous PAF plays an important role in maintenance of high pulmonary vasomotor tone and pulmonary vascular

Corresponding Author: Basil O. Ibe, Ph.D. Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, 1124 West Carson Street, RB-1, Torrance, CA 90502, Telephone number 310-222-1966, Fax: (310) 222-3887, ibe@labiomed.org.

Declaration of Interest: None

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

resistance *in utero* [6]. We have shown that PAF receptor binding and PAF receptor mRNA expression are high in lungs of fetal lambs, whereas in lungs of newly born lambs, <2 hours of age, PAF receptor binding and receptor mRNA expression were lower [7], suggesting a down-regulation of PAF receptor-mediated effects after birth. In the perinatal period, pulmonary PAF production is higher in the fetus, particularly in pulmonary veins (PV) compared to arteries (PA), however, rate of pulmonary PAF catabolism by acetylhydrolase in the fetus is slower than in the newborn [8-10]. This means that in fetal pulmonary vasculature *in vivo*, a higher PAF production coupled with a slower PAF catabolism will result in more PAF available for binding to its receptors. PAF has been implicated in the pathogenesis of hypoxia-induced pulmonary hypertension in some animal models [11,12] as well as in persistent pulmonary hypertension of the newborn (PPHN), where high circulating plasma PAF level has been measured [12].

PAF evokes its effects by binding to its G protein coupled receptor (GPCR), which is a seven trans-membrane receptor [14,15]. Studies have shown that PAF acts through its receptors to mediate pathological effects *in vivo* and *in vitro* [16-18]. PAF can also act via its receptor to evoke beneficial physiological functions [6,19]. Recently, we showed that PAF binds specifically to its receptors in ovine fetal pulmonary vein smooth muscle cells (SMC-PV) to activate specific intracellular signaling molecules, an effect that was augmented by hypoxia [20-22]. However, the effect of hypoxia on PAF binding to its receptors in adherent fetal ovine SMC-PA has not been well described. In this report, our main objective was to study PAF binding in adherent SMC-PA compared to binding in SMC-PV. Since the fetal lung is normally exposed to a low oxygen environment *in utero*, we were also interested in studying the effects of a low oxygen tension on PAF binding to its receptors in SMC-PA *in vitro*. Our hypothesis is that exposure of the cells to prolonged state of hypoxia *in vitro* will lead to greater PAF receptor protein expression and greater PAF-mediated intracellular signaling. We hope that this finding will help explain an effect of prolonged exposure of the newborn lung to hypoxia during less than chronic conditions, as may occur, *in vivo* in postnatal infants with pulmonary insufficiency. We used SMC from intrapulmonary vessels of term fetal lambs and exposed them to acute 30 min (acute) and 48 hr (prolonged) hypoxia. We measured PAF-receptor binding, PAFr mRNA expression as well as PAFr-mediated effects.

Materials and Methods

Materials

Pregnant ewes (146-148 d gestation, term being 150 d) were purchased from Nebekar Farms, Los Angeles, CA. Authentic standards of PAF: hexadecyl-2-acetyl-sn-glycerol-3-phosphorylcholine, (C₁₆-PAF); hexadecyl-sn-glycerol-3-phosphorylcholine, (Lyso-C₁₆-PAF); as well as okadaic acid, calyculin, phorbol 12-myristate 13-acetate (PMA), and genistein were purchased from Biomol Research Laboratories, Plymouth Meeting, PA. 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-glycerol-3-phosphorylcholine, 1-O-[acetyl-³H(N)]-, (³H-acetyl-C₁₆-PAF), 13.5 Ci/mmol (370 GBq/mmol). Phenylmethanesulfonyl fluoride (PMSF), leupeptin, pepstatin, bovine serum albumin (BSA), as well as antibody to alpha smooth muscle actin (α -SMA) were purchased from Sigma-Aldrich (St. Louis, MO). Antibody to PAF receptor was purchased from Cayman Chemical Company, Ann Arbor, MI. Ecolite(+) liquid scintillation cocktail was purchased from MP Biochemicals (Irvine, CA). All other reagents and chemicals were purchased from Fisher Scientific Santa Clara, CA.

Methods

Study of PAF receptors in ovine fetal pulmonary vascular smooth muscle (PVSMC)

Preparation of smooth muscle cells: Intrapulmonary vessels, 2nd to 4th generation, were isolated from near term fetal lambs and then smooth muscle cells were harvested under sterile conditions as previously reported [20]. Cells were used at 3rd to 7th passage. The identity of smooth muscle cells was established by assay with monoclonal α -SMA (SIGMA chemicals, St. Louis, MO). The cell culture medium for normoxic and hypoxic studies was Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), and 1% antibiotic and antimycotic cocktail of 0.15% nystatin, 0.15% gentamicin, and 0.01% fungizone.

Experimental Conditions

Normoxia: Smooth muscle cells from arteries (SMC-PA) and veins (SMC-PV) were studied in a humidified incubator at 37°C aerated with 5% CO₂ in air. Oxygen concentration was monitored with TED 60T percent oxygen sensor, Teledyne Analytical Instruments (City of Industry, CA). The incubator oxygen concentration was 21%. The oxygen tension (pO₂) in cell media was maintained at 80-100 Torr, and measured on a Nova Stat Profile 3 blood-gas instrument, Nova Biomedical, Waltham, MA [21,23].

Hypoxia exposure for 30 min (Acute hypoxia): The incubator was equilibrated with the hypoxia gas mixture; 2% O₂, 10% CO₂, and balance nitrogen to attain a pO₂ <40 Torr in the cell culture media. Cells were continuously aerated with the hypoxia gas mixture throughout the duration of the study, and oxygen concentration as well as oxygen tension was determined as in normoxia set up [21,23].

48 hour hypoxia (prolonged hypoxia): SMC-PA and SMC-PV were plated at cell density 1×10^4 cells/well and allowed to stabilize for 24 h. The culture medium was replaced and one set was placed in a hypoxia incubator and continuously aerated with the hypoxia gas mixture; 2% O₂, 10% CO₂, and balance nitrogen for 48 h more. The other set was placed in normoxia incubator. Oxygen concentration in hypoxia and normoxia incubators was monitored with the TED 60T percent oxygen sensor. Cell viability for the prolonged duration of hypoxia exposure was greater than 95% and was not different from viability of cells cultured in normoxia [22]. Also cell phenotype did not change because of prolonged exposure to hypoxia as determined by expression of α -SMA.

Studies of PAF receptor binding

General protocol for PAF receptor binding assay: Cells were washed with PBS and then incubated in the PAF receptor assay buffer with ³H-acetyl-C₁₆-PAF (³H-PAF) at 37°C according to the specific protocol. After incubation in normoxia or hypoxia, unbound ³H-PAF was washed off with ice-cold PBS. A mixture of 154 mM saline and 5 mM EDTA was added to the cells and incubated on ice for 30-45 min. ³H-PAF bound to its receptor was extracted on Whatman GF/C membrane filters using Whatman filter manifold and in-line vacuum system and quantified as we reported previously [20].

To study PAF interaction with its receptors in the presence of other agonist or antagonists, cells were pre-incubated with the agent or with buffer alone for control, and then ³H-PAF was added and incubated further according to the specific experimental protocol.

Specific Protocols

Specificity of PAF receptor binding in SMC-PA and SMC-PV: Studies were done as follows. a) To study PAFr binding in normoxia and hypoxia, cells were incubated with 1.0nM ^3H -PAF for 30 min in normoxia or hypoxia. b) Effect of antagonists on PAFr binding: Cells were pre-incubated for 30 min with 10 μM WEB 2170, and then 1.0nM of ^3H -PAF was added and the cells incubated for 30 min more in normoxia or hypoxia.

Effect of 48 hour (prolonged) hypoxia on PAF receptor binding: To elucidate effect of prolonged hypoxia exposure on PAFr binding to the SMC-PA and SMC PV, sub-confluent cells of each type were incubated in normoxia or hypoxia for 48 h and then PAF binding assays were conducted as described above.

Assay of PAF catabolism by the cells—Lyso-PAF was extracted from media to ascertain that differences in PAF receptor binding in SMC-PA and SMC-PV are not due to differences in PAF catabolism by the intact cells. Cells were incubated in the PAF binding assay buffer with 5nM of ^3H -PAF for 30 min in normoxia only. After incubation, the assay buffer was aspirated and subjected to lipid extraction by the Bligh and Dyer method [24], together with the ethanolic cell extracts. The cell lipid and the media extracts were concentrated with nitrogen and subjected to thin layer chromatography on silica gel G followed with high performance liquid chromatography (HPLC) purification for PAF and lyso-PAF on Ultramex C_{18} column, as we previously reported [23]. The retention times for lyso-PAF and PAF are 11.4 ± 0.3 and 15.8 ± 0.5 min respectively. One-minute HPLC fractions were collected and ^3H -PAF radioactivity co-eluting with authentic non-radiolabeled PAF standard was quantified by scintillation spectrometry (Beckman Coulter, Fullerton, CA). Amount of lyso-PAF measured was expressed in fmol/ 10^6 cells and compared statistically between catabolism by SMC-PA and SMC-PV. Amount of lyso-PAF measured (picomole lyso-PAF/min, means $\pm$ SEM, n=5) from SMC-PA was 0.196 ± 0.012 which was not different from 0.158 ± 0.018 ($p = 0.085$) measured from SMC-PV. This shows that the differences in receptor binding between SMC-PA and SMC-PV are not necessarily due to a faster PAF catabolism by SMC-PA. We then proceeded to assess PAFr protein expression by confocal microscopy and measure protein expression by Western blotting.

Western blotting for PAF receptors in PVSMC

Preparation of proteins for Western blotting: Cells used to study PAF receptor protein expression were incubated in normoxia or hypoxia in the 10% FBS culture media. To isolate proteins after incubation, cells were washed with calcium and magnesium free phosphate buffered saline and then lysed with a 40 mM HEPES lysis buffer, pH 7.4 containing a cocktail of protease inhibitors [20,23].

SDS-PAGE electrophoresis: Proteins were suspended in sodium dodecyl sulfate (SDS) sample buffer, pH 6.8, containing, 125 mM Tris-base, 4% SDS, 0.006% bromophenol blue, 36 mM EDTA, 90 mM DTT, 10% glycerol, 10 % beta-mercaptoethanol. Samples were electrophoresed for 1 h at 200V on 4-12% Tris-glycine gradient gels (Lonzo), along with Bio-Rad kaleidoscope pre-stained molecular weight markers and protein standards as we previously reported [22,23].

Study of PAF receptor protein expression by confocal microscopy—Cells were grown on sterile microscope slides to attain confluence and then washed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS. Washed cells were fixed for confocal microscopy of PAF receptor as reported previously [25] with some modifications. Briefly, cells were treated with 3% paraformaldehyde for 20 min, followed with washing with 0.1 M glycine. This was followed with three washings with PBS and then quenched by placing the slides in 10% FBS in PBS

for 30 min. Fixed cells were then incubated for 1 h at room temperature with 1:500 dilution of anti-PAF receptor antibody, diluted with 10% FBS in PBS, then washed 3× with 1% FBS in PBS. Next, cells were incubated for 30 min in 1:1000 dilution of FITC-conjugated goat anti-rabbit secondary antibody diluted with 10% FBS in PBS and then washed 3× with 1% FBS in PBS. Slides were mounted with VectaShield mounting solution with 1% propidium iodide counter stain (Vysis, Inc) and placed in -20°C until subjected to confocal microscopy within 24 h. Confocal microscopy was done on a LeicaTCS SP2 confocal microscope with krypton 65 mW, 458-488 nm/argon 25 mW, 568 nm laser and Ar/ArKr 10 mW, 633 nm laser. PAFr fluorescence was monitored under FITC filter and propidium bromide on 60× oil lenses. Another set of experiments were performed as described for the confocal study. Cells were also stained with 1:500 dilution of PAFr antibody followed with 1:1000 dilution FITC-conjugated goat anti-rabbit secondary antibodies, but without propidium iodide staining. Fluorescence images were captured on Zeiss Axioskop 40. Four to five microscope windows per slide were viewed to establish differences in PAFr protein expression in each condition.

Study of PAF receptor gene expression by real time reverse-transcription-polymerase chain reaction (rt-RT-PCR)—RNA from SMC-PA and SMC-PV of the fetal lambs were subjected to real time RT-PCR studies to determine PAF receptor expression by cells cultured in normoxia and hypoxia under baseline conditions. Total RNA was extracted with Qiagen's QIAshredder (cat #79654) and Rneasy Plus Mini Kit (cat #74134), according to the manufacturer's protocol (Qiagen, Valencia, CA). Real-time PCR was performed on ABI Prism 7000 Detection System (Applied Biosystems, Foster City, CA). Reverse transcription was accomplished with Applied Biosystems High Capacity RNA-cDNA Master Mix (cat # p/n 4390715) program as follows: step 1, 25°C for 5 min; step 2, 42°C for 30 min; step 3, 85°C for 5 min; and step 4, hold at 4°C. The cDNA was subjected to PCR with SYBR-Green PCR Master Mix (cat # p/n 4309155) as fluorescent dye, with the following steps: step 1, 50°C 1 cycle; step 2, 95°C 10 min 1 cycle; step 3, 95°C 15 sec, 59°C 1 min, 40 cycles (annealing/amplification); step 4, dissociation cure, 1 cycle, all done according to the manufacturer's protocol (Applied Biosystems) using primer pairs which are: PAFr primer #1, forward 5'- CCT GTG CAA CGT GGC TGG CT-3', bp98-117, reverse 5'- GAG ATG CCA CGC TTG CGG GT-3' bp 241-222 and PAFr primer #2, forward 5'- TCC TGT GCA ACG TGG CTG GC-3', bp 97-116, reverse 5' GAG ATG CCA CGC TTG CGG GT-3', bp 241-222, both created by using NCBI's Primer-BLAST program (<http://www.ncbi.nlm.nih.gov/tool/primer-blast/>) by entering accession number AF099674.1. Both primers sets were authenticated by RealTimePrimers.com. The GAPDH primers sequence, GenBank Accession AFO30943, at bp #108-131, and 399-375, forward 5'-ACC TGC CAA CAT CAA GTG GGG TGA T-3', reverse 5'-GGA CAG TGG TCA TAA GTC CCT CCA C-3' were synthesized by SIGMA-Aldrich (Saint Louis, MO). The negative control provided with the rt-RT-PCR kit was used according to the manufacturer's protocol (Applied Biosystems). For quantification, the target gene (PAFr gene) was normalized to GAPDH housekeeping gene used as the reference, and rt-RT-PCR data are presented as ΔC_t , means $\pm$ SD, where $\Delta C_t = C_{tT} - C_{tR}$: C_{tT} , threshold cycle of target gene (PAFr gene); C_{tR} , threshold cycle of GAPDH gene.

Study of PAF –stimulation of inositol phosphate (IP₃) release

Labeling of cells with [³H]-myo-inositol: Cells were washed with protein-free, inositol-free buffer, fed 5 μ Ci/ml of [³H]-myo-inositol in 10% FBS in inositol-free medium and incubated for 24 h in 5% CO₂ in air according to previous reports [14,20]. After 24 h in culture, medium was aspirated and cells washed with HEPES-buffered saline containing 10 mM LiCl. The medium aspirate and washes were combined and used to calculate percent

incorporation of ^3H -inositol label into cells. There was no difference in isotope incorporation between the two cell types.

Stimulation of ^3H -inositol phosphate release: Each test stimulus was prepared in the 10 mM LiCl buffer, made fresh on the day of experiment. Labeled cells were stimulated with PAF or other agents and incubated in hypoxia or normoxia for 20 min at 37°C . Reactions were quenched by adding 10 mM formic acid, and cell suspension was loaded on pre-equilibrated AG 1-X8 columns and total tritiated inositol phosphates (IP_3) were extracted as previously described [20,21]. ^3H - IP_3 radioactivity was quantified by scintillation spectrometry.

Specific protocols

PAF-stimulated inositol phosphate (IP_3) release: Cells pre-labeled with ^3H -myo-inositol were stimulated with 1nM PAF for 20 min in normoxia and hypoxia.

Specificity of PAF stimulated IP_3 release: To test the specificity of PAF receptor-mediated inositol phosphate production, cells pre-labeled with ^3H -myo-inositol for 24 hr in inositol-free medium were pre-incubated for 20 min in normoxia and hypoxia with $10\mu\text{M}$ of the PAF receptor antagonist WEB 2170, with $30\mu\text{M}$ genistein, or with buffer for controls. Then 1.0nM of PAF was added and incubated for 20 min more. The reactions were quenched by adding 10mM formic acid, incubating in ice bath for 30 min followed with neutralization with 15mM ammonium hydroxide

Effect of PMA on PAF-stimulated IP_3 release: As a GPCR, PAF binding to its receptor is subject to regulation of its signal transduction pathways by processes involving activation as well as desensitization of the receptor. We investigated the possible desensitization of PAF receptor by phorbol myristyl acetate (PMA), a GPCR linked PKC activator. Cells at 40%-50% confluence were first cultured in normoxia or hypoxia for 48 h in inositol-free DMEM containing $10\mu\text{Ci/ml}$ of ^3H -myo-inositol. After a total of 48 h in hypoxia, cells were stimulated for 5 min with 100nM PMA in hypoxia. PMA was washed off with HEPES-buffered saline and then studied for concentration effect of PAF (nM: 1, 10, and 100) release of inositol phosphates by incubating the cells in normoxia for 20 min.

PAF stimulation of Ca^{2+} release by SMC-PA—We have reported that PAF and hypoxia augmented intracellular Ca^{2+} (calcium) release in SMC-PV [20]. In this protocol, we investigated effect of PAF and hypoxia on intracellular Ca^{2+} release in SMC-PA only. Calcium release was measured with Fluo-4 NW fluorescence indicator kit (Molecular Probes) following the instructions provided by the vendor. Cells were grown in hypoxia for 48 h in 4-chamber slides till confluent and then the chambers were washed once with PBS assay buffer provided with the kit. Fluo-4 NW was added, $240\mu\text{l/chamber}$, and cells were incubated in hypoxia and normoxia for 1 h more. Then 10nM PAF was added, incubated for 30 min, and then aspirated. Cell counts were determined before the cells were lysed with $100\mu\text{l}$ lysis buffer provided by the vendor. Lysed cells were transferred to 96-well plate for measurement of Fluorescein fluorescence (485/535nm).

Data analysis and statistics: All numerical data are means $\pm$ SEM, means $\pm$ SD for rt-RT-PCR data. Where radioisotope was used and unless indicated otherwise, the background radioactivity was subtracted before quantifying the amount of radioactivity. Then normalized data were analyzed with two-tailed t-test followed with ANOVA and Tukey posthoc test (Prism program). Results were considered as significant at $p < 0.05$

Results

Figure 1 shows the specific PAFr binding in SMC-PA and SMC-PV during normoxia and acute hypoxia. Data are in fmol/ 10^6 cells. In figure 1a, for cells cultured in normoxic conditions, binding in SMC-PA was 5.16 ± 0.65 , which increased significantly to 7.70 ± 0.36 when cells were exposed to 30 min hypoxia. In SMC-PV, binding in normoxia was 19.33 ± 1.08 which increased significantly to 27.78 ± 1.58 when cells were exposed to 30 min hypoxia. Thus when cells were grown in normoxia, acute hypoxia exposure (30 min) enhanced PAF receptor binding in both cell types, but binding in SMC-PV was greater than binding in SMC-PA both in normoxia and hypoxia.

Figure 1b shows the effect of 48 h hypoxia (prolonged hypoxia) exposure on PAF receptor binding. In SMC-PA, when cells from prolonged hypoxia were exposed to 30 min normoxia, binding was 14.80 ± 0.31 , whereas receptor binding in all hypoxia conditions was 37.40 ± 2.1 . Thus exposure of cell to 30 min normoxia after prolonged hypoxia decreased PAFr binding compared to an all hypoxia study (figure 1b). The corresponding values for SMC-PV are 27.70 ± 1.70 for cells studied in 30 min normoxia after 48 h hypoxia and 54.07 ± 3.02 for cells studied in all hypoxic conditions. Therefore 48 hr hypoxia significantly increased PAFr binding compared to cells grown in normoxia and studied in normoxia, figure 1a. Exposure of cells from 48 h hypoxia to 30 min of normoxic conditions decreased binding compared to cells studied under all hypoxic conditions. When SMC-PV cultured in 48 h hypoxia was exposed to 30 min normoxic conditions, receptor binding was comparable to effect obtained when SMC-PV cultured in normoxia was exposed to 30 min acute hypoxia. In both SMC-PA and SMC-PV, pretreatment of cells with $10\mu\text{M}$ of the PAF receptor antagonist WEB 2170, decreased PAF binding to 3.7 ± 0.47 in normoxia and hypoxia in both cell types showing a PAF receptor specific binding even after 48 h exposure to hypoxia.

Figure 2 shows the effect of 48 h hypoxia on PAF receptor binding normalized to PAF binding to cells cultured in normoxia and studied in 30 min normoxic conditions. The figure shows that 48 h hypoxia caused 7.2-fold increase in PAF binding to SMC-PA, but only 2.8-fold increase in binding to SMC-PV. Thus prolonged hypoxia of smooth muscle cells in culture modulates PAF effect more in SMC-PA than in SMC-PV.

Figure 3 shows qualitative confocal microscopy to examine PAF receptor protein expression. In SMC-PA, figure 3A and SMC-PV, figure B, cells were incubated in normoxia, upper row or hypoxia, lower row, without stimulation. The photomicrographs were captured under the same gain and they are on the same plane. In figures 3C and 3D, incubation of cells in hypoxia augmented PAFr fluorescence and addition of 10nM PAF for 2 hr increased PAFr fluorescence above hypoxia alone in both cell types. Four to five different microscope views were qualitatively examined for PAFr signal. In both SMC-PA and SMC-PV, PAF receptor signals are much brighter in hypoxia than in normoxia.

Figure 4 shows the Western blotting of PAF receptor protein expression by SMC-PA (Panel A) and SMC-PV (Panel B) studied with and without 10nM PAF in normoxia and after 48 h hypoxia (prolonged hypoxia). Receptor protein expression was quantified by protein loading and relative to total actin expression. In both cell types, treatment of cells with 10nM PAF in normoxia increased PAF receptor protein expression. Also in both cell types, hypoxia increased PAFr protein expression and addition of 10nM PAF further increased receptor protein expression.

Figure 5 shows PAF receptor (PAFr) mRNA expression by SMC-PA and SMC-PV of the fetal lambs under un-stimulated conditions in normoxia and hypoxia. The mRNA expression is normalized to expression of the GAPDH internal standard. In both SMC-PA and SMC-

PV, hypoxia significantly increased PAFr mRNA expression. However PAFr mRNA expression by SMC-PV was lower than expression by SMC-PA under both conditions.

Figure 6 shows the effect of PAF on inositol phosphate release (IP_3 DPM (10^3)/ 10^6 cells) by cells culture in normoxia and studied in 20 min normoxia or hypoxia. In both SMC-PA and SMC-PV, baseline inositol phosphate release was very low and was subtracted before statistical analysis. In SMC-PA, treatment of cells with 1nM PAF released 1345 ± 310 during normoxia and 2750 ± 200 during hypoxia. PAF-stimulated inositol phosphate release was more in hypoxia compared to normoxia. In SMC-PV, inositol phosphate release was 5400 ± 100 in normoxia, which increased to 8650 ± 300 in hypoxia. Inositol phosphate release in hypoxia was greater than release in normoxia. Compared to SMC-PA, inositol phosphate release by SMC-PV was 4-fold greater than SMC-PA release in normoxia and 3-fold greater than SMC-PA release in 30 min hypoxia. In both SMC-PA and SMC-PV, PAF-stimulated inositol phosphate release was inhibited by the PAF receptor antagonist WEB 2170 and by genistein, a tyrosine kinase inhibitor indicating pathway-specific PAF stimulation of inositol phosphate release.

Figure 7 shows the effect of 5-min PMA pre-treatment on PAF-stimulated inositol phosphate release ($DPM \times 10^3/10^6$ cells) by SMC-PA (Panel A) and SMC-PV (Panel B) after 48 h exposure of cells to hypoxia. In SMC-PA (Panel A), baseline inositol phosphate release was 36.5 ± 2.8 , which increased to 44.1 ± 1.4 on treatment with 1nM PAF, and increased further on stimulation with 10nM PAF. Stimulation with 100nM PAF decreased inositol phosphate release but was comparable to release by 10nM PAF. In each instance, including baseline conditions, pretreatment of cells with 100nM PMA for 5 min caused significant attenuation in PAF stimulation of inositol phosphate release. In SMC-PV (Panel B), baseline inositol phosphate release was 4.1 ± 0.3 which increase to 12.9 ± 1.0 on stimulation with 1nM PAF. Stimulation with 10nM PAF increased inositol phosphate release over 1nM PAF and stimulation with 100nM PAF produced further increase in release over 10nM PAF treatment. In SMC-PV, stimulation of cells with PAF produced a concentration dependent release of inositol phosphate by the cells. In both cell types, pretreatment of cells with 100nM PMA for 5 min before stimulation with PAF caused significant attenuation in PAF-stimulated inositol phosphate release. It is noteworthy that baseline inositol phosphate release by SMC-PA after 48 h hypoxia exposure is 9-fold greater than baseline inositol phosphate release by SMC-PV after 48 h hypoxia exposure, suggesting an exaggerated effect of prolonged hypoxia in SMC-PA.

Figure 8 show effect of PAF and hypoxia on calcium release by SMC-PA in units of calcium fluorescence normalized to 2×10^3 cells. Under baseline conditions, calcium fluorescence was 1831 ± 27 . Hypoxia alone increased calcium release. Stimulation of cells with 10nM PAF in normoxia or hypoxia significantly increased calcium release over baseline conditions in normoxia or hypoxia. Thus PAF and hypoxia augmented calcium release by SMC-PA, and as we reported previously [20], PAF and hypoxia also augments calcium release by SMC-PV.

Discussion

Platelet activating factor is a phospholipid with a wide range of biological activities [1,2], including action as a potent vasoconstrictor in the pulmonary circulation [3,4,6]. Previous studies have shown that PAF binds to its receptor to induce intracellular signaling in ovine fetal intrapulmonary venous smooth muscles cells [20,21,22]. PAF receptor binding in pulmonary vascular SMC of arterial origin has not been well described. Moreover, effect of prolonged condition of hypoxia on PAF-mediated effects has not been characterized. In this study, we investigated effect of oxygen tension on PAF binding to its receptors in SMC-PA

and compared the effect on SMC-PV. We found that hypoxia up-regulates PAF receptor binding as well as PAF receptor protein expression in both cell types, and quantitatively, hypoxia enhanced PAF binding to SMC-PV more than binding to SMC-PA, even though the relative effect of prolonged hypoxia on PAFr binding to SMC-PA was greater. Greater PAF binding to SMC-PV suggests greater PAFr mRNA expression, and/or greater posttranslational regulation of PAFr protein expression in SMC-PV. However, when we investigated PAFr mRNA expression by the two cell types employing real time RT-PCR, we found that PAFr mRNA expression by SMC-PA was more than by SMC-PV during both normoxia and hypoxia. In a previous report [20], we showed that significant autophosphorylation of PAF receptor occurs in SMC-PV both in normoxia and hypoxia, and treatment of the cells with PAF augments the receptor phosphorylation. It was also demonstrated that increase in phosphorylation correlated with the increase in PAFr-mediated effects [20]. Thus although, in this study, PAFr mRNA expression was greater in SMC-PA, our findings indicate that translational as well as posttranslational modifications of the PAFr, for instance, receptor phosphorylation may exert more effect on PAFr levels and PAFr-mediated effects. Acute or prolonged hypoxia increased the effect of PAF on the response that was being measured, suggesting adverse consequences of superimposition of state of hypoxia on the pathogenetic effects of PAF, which is an endogenous lipid metabolite. Thus in neonates with compromised pulmonary ventilation and perfusion, oxygenation will attenuate adverse PAFr-mediated effects. This presents an interesting phenomenon that implicates the reversibility of effect of hypoxia on PAF-PAFr interactions. PAFr is a member of the G-protein coupled receptors (GPCR), a family of proteins with seven transmembrane loops [14,15]. We can speculate that the decrease in PAFr effect in normoxia suggests the occurrence of an immediate auto-dephosphorylation of the receptor in normoxic conditions. Western analysis of PAF receptor protein expression in normoxia and hypoxia showed higher expression of receptor protein during hypoxia in both cell types, in support of the greater PAF receptor binding in hypoxia. In an unrelated study [27], chronic myeloid leukemia cells incubated in 1% oxygen for 8 days showed increased PAFr expression. The relevance of this finding to our study is that it supports our finding that PAFr expression is inducible by hypoxia. In this study, though both 30-min and prolonged exposure to hypoxia enhanced PAFr binding in both cell types, absolute PAFr binding was always greater in SMC-PV, but quantitatively, prolonged hypoxia produced greater binding when compared to a normalized effect of 30 min normoxia on both cell types. This suggests that exposure of the newborn lung to prolonged hypoxia, post-natally, will induce greater PAF receptor expression and binding in SMC of pulmonary arteries which are crucial in postnatal pulmonary circulation. This increased PAFr binding may lead to onset of persistent pulmonary hypertension of the newborn.

Signaling characteristics of PAF receptor in SMC-PA and SMC-PV

PAF receptor protein expression studied by confocal microscopy showed higher PAF receptor expression in hypoxia. The confocal microscopy shows some heterogeneity in PAF receptor protein expression in the cells as has been previously observed in vascular endothelium [25]. Thus we can speculate that *in vivo*, in the hypoxic environment of fetal lungs, pulmonary vessels, especially veins, will express more PAF receptor protein, and that postnatal exposure of the newborn lung to prolonged state of hypoxia will predispose the newborn lung to greater PAFr-mediated effects. Administration of PAF receptor antagonist *in vivo* to fetal and newborn lambs decreased the high pulmonary vasomotor tone and improved lung blood flow in the fetus and newborn lambs indicating involvement of PAFr in the high vasomotor tone [6]. Smooth muscle cells of fetal lambs exposed to long term high altitude hypoxia *in utero* also show exaggerated PAFr protein expression and proliferative potential compared to cells from control lambs [28], suggesting involvement of PAFr-mediated signaling in the pulmonary vascular remodeling observed in these lambs.

Our data on prolonged exposure of SMC to hypoxia bears some semblance to effect of chronic in utero hypoxia of the fetal lambs thus implying that in vivo, prevention of PAF interaction with its receptor may present one method to ameliorate hypoxia induced adverse PAFr-mediated pulmonary abnormalities.

PAF receptor binding activates phosphoinositide phospholipase C, with release of IP₃ and subsequent [Ca²⁺]_i mobilization [14,15,18,29]. In this study, PAF and hypoxia up-regulated the PAF-mediated release of IP₃ showing that the PAF receptors in the cells are functional. When we tested for specificity, PAF-stimulated IP₃ production was significantly inhibited by WEB 2170, a PAF-receptor antagonist, indicating that IP₃ release occurred by specific PAF receptor-mediated mechanisms, and by genistein indicating a protein kinase (PK) C and tyrosine kinase-mediated pathway. Protein kinases such as PKC, PKA and PKG are believed to play important roles in contractility and Ca²⁺-desensitization in smooth muscle, but the pathway of this desensitization is not fully understood [30]. PKC activation is an important regulator of GPCR-linked hydrolysis of phosphoinositide and calcium flux in different types of SMC [30-33]. In canine tracheal SMC, pre-treatment of cells with PMA attenuated ability of 5-hydroxytryptamine to stimulate phosphoinositide hydrolysis [31] and PKC is involved in desensitization of mu-opioid receptor in brain neurons [32]. In this report, we show that in SMC-PA and SMC-PV, 5 min pre-treatment of cells with the PKC activator, PMA decreased PAF-stimulated IP₃ release suggesting involvement of PKC/PLC in PAFr-stimulated inositol phosphate release and desensitization of the PAFr by pre-exposure to PMA. In a previous study, we showed that PAF stimulation of intracellular Ca²⁺ mobilization is increased by hypoxia [20]. In this report, we show that PAF stimulates Ca²⁺ flux in SMC-PA and the condition of hypoxia augments this PAF-mediated effect. In a study employing PAF receptor knockout (PAFR^{-/-}) mice, it was observed that the PAFR^{-/-} mice lacked long-term ventilatory facilitation when subjected to prolonged intermittent hypoxia. This suggests the involvement PAF receptor at the respiratory center of the PAFr knockout controls normoxic ventilation [34]. In this study, we speculate, based on our findings, that following exposure to chronic hypoxia in the fetus, or prolonged hypoxia post-natally, PAF and PAFr-mediated effects will be exaggerated and are likely to be greater in the pulmonary arteries than in veins, resulting in greater vasoconstriction and remodeling in the arteries. We can further infer from our present findings that exposure of the newborn lungs to prolonged state of hypoxia will result in increased PAF receptor binding together with increased release of IP₃ by PAF, independently or in conjunction with a relevant endogenous mediator such as endothelin-1, will lead to increased intracellular calcium levels in the cells.

Acknowledgments

This study was supported by the National Institutes of Health, Bethesda, MD [Grant HL-077819]; and the Los Angeles Biomedical Research Institute, Torrance, CA [Grant 513292]. The authors are grateful for help in real time RT-PCR studies provided by Peng Xia of the Gunther Core Molecular Biology laboratories at the Los Angeles Biomedical Research Institute.

References

1. Ishii S, Shimizu T. Platelet-activating factor (PAF) receptor and genetically engineered PAF receptor mutant mice. *Prog lipid Res* 2000;39:41–82. [PubMed: 10729607]
2. Stafforini DM, McIntyre TM, Zimmerman GA, Prescott SM. Platelet-activating factor, a pleiotrophic mediator of physiological and pathological processes. *Crit Rev Clin Lab Sci* 2003;40:643–672. [PubMed: 14708958]
3. Raj JU, Toga H, Ibe BO, Anderson J. Effects of endothelin, platelet activating factor and thromboxane A₂ in ferret lungs. *Respir Physiol* 1992;88:129–140. [PubMed: 1626132]

4. Toga H, Ibe BO, Raj JU. Vascular effects of platelet-activating factor in lambs: role of cyclo- and lipoxygenase. *J Appl Physiol* 1992;73:2559–2566. [PubMed: 1490969]
5. Gao Y, Zhou H, Raj JU. PAF induces relaxation of pulmonary arteries but contraction of pulmonary veins in the ferret. *Am J Physiol Heart Circ Physiol* 1995;269:H704–H709.
6. Ibe BO, Hibler S, Raj JU. Platelet-activating factor modulates pulmonary vasomotor tone in the perinatal period. *J Appl Physiol* 1998;85:1079–1085. [PubMed: 9729586]
7. Ibe BO, Sander FC, Raj JU. Platelet-activating factor receptors in lamb lungs are down-regulated immediately after birth. *Am J Physiol Heart Circ Physiol* 2001;278:H1168–H1176. [PubMed: 10749711]
8. Salva AML, Ibe BO, Cliborn E, Reyes G, Raj JU. Hypoxia attenuates metabolism of platelet activating factor by fetal and newborn lamb lungs. *J Lipid Res* 1992;37:783–789. [PubMed: 8732778]
9. Ibe BO, Pham HH, Kääpä P, Raj JU. Maturational changes in ovine pulmonary metabolism of platelet-activating factor: Implications for postnatal adaptation. *Mol Genet Metab* 2001;74:385–395. [PubMed: 11708870]
10. Ibe BO, Sander FC, Raj JU. Platelet activating factor acetylhydrolase activity in lamb lungs is up-regulated in the immediate newborn period. *Mol Genet Metab* 2000;69:46–55. [PubMed: 10655157]
11. Ono S, Westcott JY, Voelkel NF. PAF antagonists inhibit pulmonary vascular remodeling induced by hypobaric hypoxia in rats. *J Appl Physiol* 1992;73:1084–1092. [PubMed: 1400021]
12. Chen D, Chen W. Changes in distribution of platelet activating factor in lung of rats with hypoxic pulmonary hypertension. *Chin Med J (Eng)* 1996;109:776–779.
13. Caplan MS, Hsueh W, Sun XM, Gidding SS, Hageman JR. Circulating plasma platelet activating factor in persistent pulmonary hypertension of the newborn (PPHN). *Am Rev Respir Dis* 1990;142:1258–1262. [PubMed: 2252241]
14. Carlson SA, Chatterjee TK, Fisher RA. The third intracellular domain of the platelet-activating factor receptor is a critical determinant in the receptor coupling to phosphoinositide phospholipase C activating G proteins. *J Biol Chem* 1996;272:23146–23153. [PubMed: 8798508]
15. Parent JL, Le Gouill C, Escher E, Rola-Pleszcynski M, Stankova J. Identification of transmembrane domain residues determinant in the structure-function relationship of human platelet-activating factor receptor by site directed mutagenesis. *J Biol Chem* 1996;271:23298–23303. [PubMed: 8798529]
16. Ishii S, Nagase T, Shindou H, Takizawa H, Ouchi Y, Shimizu T. Platelet-activating factor receptor develops Airway hyperresponsiveness independently of airway inflammation in murine asthma model. *J Immunol* 2004;172:7095–7102. [PubMed: 15153532]
17. Uhlig S, Göggel R, Engel S. Mechanisms of platelet-activating factor (PAF)-mediated responses in the lung. *Pharmacol Rev* 2005;57(suppl):206–221.
18. Aponte M, Jiang W, Lakkis M, Li MJ, Edwards D, Albitar L, Vitonis A, Mok SC, Cramer DW, Ye B. Activation of PAF-receptor and pleiotropic effects on tyrosine phospho-EGFR/Src/FAK/Paxillin in ovarian cancer. *Cancer Res* 2008;68:5839–5848. [PubMed: 18632638]
19. Brewer C, Bonin F, Bullock P, Nault MC, Morin J, Imbeault S, Shen TY, Franks DJ, Bennett SAL. Platelet activating factor-induced apoptosis is inhibited by ectopic expression of the platelet activating factor G-protein coupled receptor. *J Neurochem* 2002;82:1502–1511. [PubMed: 12354298]
20. Ibe BO, Portugal AM, Chaturvedi S, Raj JU. Oxygen-dependent PAF receptor binding and intracellular signaling in ovine fetal pulmonary vascular smooth muscle. *Am J Physiol* 2005;288:L879–L886.
21. Ibe BO, Ameer A, Portugal AM, Renteria L, Raj JU. Platelet-activating factor modulates activity of cyclic nucleotides in fetal ovine pulmonary vascular smooth muscle. *J Pharmacol Exp Ther* 2007;320:728–737. [PubMed: 17085546]
22. Ibe BO, Abdallah MF, Portugal AM, Raj JU. Platelet-activating factor stimulates ovine foetal pulmonary vascular smooth muscle cell proliferation: role of nuclear factor-kappa B and cyclin-dependent kinases. *Cell Prolif* 2008;41:208–229. [PubMed: 18336468]

23. Ibe BO, Portugal, Raj JU. Metabolism of platelet activating factor by intrapulmonary vascular smooth muscle cells: effect of oxygen on phospholipase A₂ protein expression and activities of acetyl-CoA acetyltransferase and cholinephosphotransferase. *Mol Genet Metab* 2002;77:237–248. [PubMed: 12409272]
24. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959;37:911–917. [PubMed: 13671378]
25. Ihida K, Pradescu D, Czekay RP, Palade GE. Platelet-activating factor receptor (PAF-R) is found in a large endosomal compartment in human umbilical vein endothelial cells. *J Cell Sci* 1999;112:285–295. [PubMed: 9885282]
26. Velasquez LA, Ojeda SR, Croxatto HB. Expression of platelet-activating factor receptor in the hamster oviduct: localization to the edosalpinx. *J Reprod Fertil* 1996;109:3249–3354.
27. Desplat V, Faucher JC, Mahon FX, Sharba PD, Praloran V, Ivanovic Z. Hypoxia modifies proliferation and differentiation of CD34⁺ CML cells. *Stem Cells* 2002;20:347–354. [PubMed: 12110704]
28. 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* 2007;293:L1475–L1482. [PubMed: 17951313]
29. Lin AY, Rui YC. Platelet-activating factor induced calcium mobilization and phosphoinositide metabolism in cultured bovine cerebral microvascular endothelial cells. *Biochim Biophys Acta* 1994;1224:323–328. [PubMed: 7981248]
30. Ihara E, MacDonald JA. The regulation of smooth muscle contractility by zipper-interacting protein kinase. *C J Physiol Pharmacol* 2007;85:79–87.
31. Yang CM, Fen LW, Tsao HL, Chiu CT. Inhibition of 5-hydroxytryptamine-induced phosphoinositide hydrolysis and Ca²⁺ mobilization in canine cultured tracheal smooth muscle cells by phorbol ester. *Br J Pharmacol* 1997;121:853–860.
32. Bailey CP, Oldfield S, Llorente J, Caunt CJ, Teschemacher AG, Roberts L, McArdle CA, Smith FL, Dewey WL, Kelly E, Henderson G. Involvement of PKC α and G-protein-coupled receptor kinase 2 in agonist-selective desensitization of μ -opioid receptors in mature brain neurons. *Br J Pharmacol* 2009;158:157–164. [PubMed: 19309357]
33. Deshpande DA, Pascual RM, Wang SW, Eckman DM, Reimer EC, Funk CD, Penn RB. PKC-dependent regulation of the receptor locus dominates functional consequences of cysteinyl leukotriene type 1 receptor activation. *FASEB J* 2007;21:2335–2342. [PubMed: 17392478]
34. Reeves SR, Gozal D. Platelet-activating factor receptor modulates respiratory adaptation to long-term intermittent hypoxia in mice. *Am J Physiol Regul Integr Comp Physiol* 2004;287:R369–R374.

Figure 1a

Figure 1b

Figure 1.

Figure 1a Effect of 30 min (acute) hypoxia on PAF receptor binding in SMC-PA and SMC-PV. Data are means $\pm$ SEM, $n = 5$. Cells were cultured in normoxia and then incubated for 30 min in hypoxia as described in methods section. PAFr binding in SMC-PV was greater than binding in SMC-PA however, hypoxia increased PAF binding in both SMC-PA and SMC-PV. The statistics are: * $p < 0.05$, different from normoxia; ** $p < 0.05$, different from SMC-PA in normoxia or hypoxia.

Figure 1b Effect of 48 h hypoxia (prolonged hypoxia) on PAF binding to its receptors in SMC-PA and SMC-PV. Data are means $\pm$ SEM, $n = 6$. Cells were cultured for 48 hr in hypoxia and studied for PAF receptor binding in normoxia for 30 min or for 30 min more in

hypoxia. Prolonged hypoxia significantly increased PAF binding to its receptors and then binding in SMC-PV was still greater than binding in SMC-PA. PAF binding in normoxia or hypoxia was attenuated by the PAF receptor antagonist WEB 2170. The statistics are: * $p < 0.05$, different from normoxia; ** $p < 0.05$, different from binding in SMC-PA; + $p < 0.05$, different from control.

Figure 2.

PAF receptor binding to SMC-PA and SMC-PV in prolonged hypoxia normalized to binding in 30 min (acute) normoxic conditions.

Effect of prolonged hypoxia on PAF receptor binding compared to binding in 30 min (acute) normoxia conditions. Data are means $\pm$ SEM, $n = 6$. The ratio of PAF binding after 48 h hypoxia to binding after 30 min normoxia was calculated and presented as fold increase in binding. In both SMC-PA and SMC-PV, 48 h hypoxia significantly increased PAF binding. The statistics are: * $p < 0.05$, different from 48 hr normoxia; ** $p < 0.05$, different from effect of prolonged conditions G11 on SMC-PA.

Figure 3A, SMC-PA; confocal images **Figure 3C SMC-PA, fluorescence images**

Figure 3B, SMC-PV confocal images **Figure 3D, fluorescence images** **Figure 3.**

Qualitative confocal microscopy (figure 3A) and fluorescence microscopy (figure 3C) of effect of hypoxia on PAF receptor protein expression in SMC-PA. Qualitative confocal microscopy (figure 3B) and fluorescence microscopy (figure 3D) of effect of hypoxia on PAF receptor protein expression in SMC-PV. Composites of confocal micrographs of PAF receptor protein expression by SMC-P, figure 3A and SMC-PV, figure 3B. Cells were prepared for confocal microscopy as described in methods. The images were obtained under the same gain and axis. In both figures 3A and 3B, the left subpanel shows the staining of PAF receptor in the soma, the middle subpanel shows the propidium iodide (PI) nuclear stain, and the right subpanel is the composite of the

soma and nuclear staining. In general, hypoxia enhanced the PAFr expression in both cell types.

Figure 4. Western blotting of PAF receptor protein expression by SMC-PA (Panel A) and SMC-PV (Panel B) studied with and without 10nM PAF in normoxia and prolonged hypoxia, Western analysis of PAFr protein expression by the two cell types. Studies were done as described in methods in normoxia and prolonged hypoxia. The lanes are as identified. Numerical data are means $\pm$ SEM, $n = 3$ different determinations. In both SMC-PA and SMC-PV, 10nM PAF enhanced PAFr protein expression in normoxia and hypoxia. The statistics are * $p < 0.05$, different from control in both SMC-PA and SMC-PV; ** $p < 0.05$, different from control in both SMC-PA and SMC-PV; and # $p < 0.05$, different from all other conditions in normoxia and hypoxia.

Figure 5.

Real time RT-PCR of PAF receptor mRNA expression by SMC-PA and SMC-PV studied under baseline conditions.

Real-time RT-PCR analysis of PAFr mRNA expression by SMC-PA and SMC-PV studied in baseline conditions during normoxia and hypoxia. Data are means $\pm$ SD, $n = 4$ different studies run in triplicates. Both cell types expressed PAFr mRNA in normoxia and culture of cells in hypoxia increased PAFr mRNA expression by both cell types. The statistics are: * $p < 0.05$, different from SMC-PA; # $p < 0.05$, different from SMC-PA in normoxia and hypoxia.

Figure 6.

Effect of acute hypoxia on PAF stimulation of inositol phosphates (IP₃) release. Effect of hypoxia on PAF-stimulated inositol phosphate (IP₃) release by SMC-PA and SMC-PV. Data are means $\pm$ SEM, n=6. Cells were pre-incubated for 20 min with 10 μ M WEB 2170 or 10 μ M genistein and studied as described in methods section. In both SMC-PA and SMC-PV studies, baseline radioactivity was subtracted before statistical analysis. PAF stimulated the release of IP₃ in normoxia and hypoxia. Inositol phosphate release by SMC-PV was greater than release by SMC-PA. PAF-stimulated IP₃ release was inhibited by WEB 2170 and by genistein. The statistics are: *p < 0.05, different from normoxia; **p < 0.05, different from PAF effect in SMC-PA; #p < 0.05, different from effect of PAF alone.

Panel A

Panel B

Figure 7.

Effect of 5-min PMA pre-treatment on PAF-stimulated inositol phosphate release ($\text{DPM} \times 10^3/10^6$ cells) by SMC-PA (panel A) and SMC-PV (Panel B) after prolonged exposure of cells to hypoxia.

Effect of 48 h hypoxia (prolonged hypoxia) and PMA on PAF-stimulated release of inositol phosphates by SMC-PA and SMC-PV. Data are means $\pm$ SEM, $n = 5$. After prolonged hypoxia, cells were stimulated with buffer alone, with 100nM PMA, or with 1nM, 10nM, and 100nM PAF and incubated for 20 min in hypoxia. PAF stimulation of IP_3 release was concentration dependent. Pre-treatment of cells for 5 min with 100nM PMA attenuated PAF stimulation of IP_3 release. The statistics are: * $p < 0.05$, different from buffer and preceding PAF concentration; # $p < 0.05$, different from PAF alone.

Figure 8. Effect of PAF and hypoxia on calcium release by SMC-PA in units of calcium fluorescence. Effect of hypoxia and 10nM PAF on calcium flux in SMC-PA. Data are means $\pm$ SEM, n = 4. Both hypoxia and PAF increased intracellular calcium flux in SMC-PA. The statistics are: *p < 0.05, different from normoxia; #p < 0.05, different from control.