Platelet-activating factor stimulates ovine foetal pulmonary vascular smooth muscle cell proliferation: role of nuclear factor-kappa B and cyclin-dependent kinases

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Abstract. *Objective:* Platelet-activating factor (PAF) is implicated in pathogenesis of persistent pulmonary hypertension of the neonate (PPHN); PAF is a mitogen for lung fibroblasts. PAF's role in pulmonary vascular smooth muscle cell (PVSMC) proliferation and in hypoxia-induced pulmonary vein (PV) remodelling has not been established and mechanisms for PAF's cell-proliferative effects are not well understood. We investigated involvement of PAF and PAF receptors in PVSMC proliferation. *Materials and methods:* Cells from pulmonary arteries (SMC-PA) and veins (SMC-PV) were serum starved for 72 h in 5% CO₂ in air (normoxia). They were cultured for 24 h more in normoxia or 2% O₂ (hypoxia) in 0.1% or 10% foetal bovine serum with 5 µCi/well of [³H]-thymidine, with and without 10 nM PAF. Nuclear factor-kappa B (NF-κB), CDK2 and CDK4 protein expression, and their roles in cell proliferation control were studied. *Results:* PAF and hypoxia increased SMC-PA and SMC-PV proliferation. WEB2170 inhibited PAF-induced cell proliferation while lyso-PAF had no effect. SMC-PV proliferated more than SMC-PA and PAF plus hypoxia augmented NF-κB protein expression. NF-κB inhibitory peptide attenuated PAF-induced cell proliferation by 50% and PAF increased CDK2 and CDK4 protein expression. The data show that hypoxia and PAF up-regulate PVSMC proliferation *via* PAF receptor-specific pathway involving NF-κB, CDK2 and CDK4 activations. *Conclusion:* They suggest that *in vivo*, in foetal lung low-oxygen environment, where PAF level is high, proliferation of PVSMC will occur readily to modulate PV development and that failure of down-regulation of PAF effects postnatally may result in PPHN.

INTRODUCTION

Cell proliferation *in vivo* and *in vitro* can be initiated by endogenous and exogenous stimuli, and induction of cell growth may involve autocrine or paracrine mechanisms (Bussolino & Camussi 1995; Rose *et al.* 1995). Growth factors and cytokines produced *in vivo* under normal conditions, can interact by paracrine mechanisms to induce and sustain vascular growth (Berk 2001). However, uncontrolled proliferation of vascular smooth muscle cells *in vivo* is one of the

fundamental signs of vascular diseases such as atherosclerosis and neonatal pulmonary hypertension (Schwartz *et al.* 1990; Bussolino & Camussi 1995; Chabrier *et al.* 1996; Berk 2001). Platelet-activating factor (PAF) is an autocoid that, depending on the stimulus and physiological conditions of the local cellular environment, can evoke beneficial or deleterious effects in pulmonary physiology and pathophysiology (Ibe *et al.* 1998; Stafforini *et al.* 2003). We have shown that in foetal lamb lungs, PAF receptor-binding and PAF receptor mRNA expression are high, so that a high level of pulmonary vasomotor tone can be maintained *in utero* (Ibe *et al.* 2000), whereas in lungs of the newly born lamb, PAF receptor-binding and PAF receptor mRNA expression are low, suggesting the existence of a mechanism for down-regulating PAF receptor-mediated effects *in vivo* (Ibe *et al.* 2000), to prevent deleterious effects of PAF postnatally. We have recently shown that hypoxia increases PAF receptor-binding and PAF receptor protein expression in foetal ovine pulmonary vascular smooth muscle cells (PVSMC), indicating a physiological correlation between *in vivo* status of PAF–PAF receptor effects and *in vitro* conditions (Ibe *et al.* 2005). PAF stimulates proliferation of SMCs of systemic origin (Heery *et al.* 1995; Gaumond *et al.* 1997; Fischer *et al.* 2006; Ninio 2006) *via* pathways involving its G-protein-coupled receptor. In rats, PAF stimulates growth of aortic SMCs *in vivo* and *in vitro* (Raisanen *et al.* 1993) and PAF stimulates growth of human bronchial SMCs in culture (Ibe *et al.* 2006). Recently, we showed that PAF induces proliferation of pulmonary venous smooth muscle cells in culture *via* an epidermal growth factor receptor (EGFR)-linked pathway (Zhou *et al.* 2007). These data show that PAF also has potent mitogenic effects on PVSMCs. However, mechanisms of the mitogenic effect of PAF on PVSMCs are not known.

We are interested in understanding the mechanisms of foetal pulmonary vascular growth *in utero* and in the consequences of uninhibited cell growth postnatally. Our primary hypothesis in this study is that in the low-oxygen environment of the foetus *in utero*, PAF binding to its receptors induces pulmonary vascular smooth muscle cell proliferation resulting in increased vascularization, whereas in the newborn, abnormal stimulation of cell proliferation is indicative of pulmonary vascular abnormality, such as neonatal pulmonary hypertension. Scheme 1 describes the hypothesis that we investigated. We hypothesize that PAF binding to its G-protein-coupled receptor activates the PAF receptor by inducing its own phosphorylation (PAFr-P). The phosphorylated PAF receptor then induces phosphorylation of MAPK p44/p42 (Erk1/2), which in turn induces translocation of cytosolic nuclear factor-kappa B (NF- κ B) to the nucleus directly or *via* activation of other intracellular signalling proteins. In the nucleus, NF- κ B initiates a chain of events that includes activation of cyclin-dependent kinases (CDK2 and CDK4) and phosphorylation of the retinoblastoma (Rb) protein. Phosphorylated Rb protein then stimulates gene expression and cell proliferation. We used ovine foetal intra-PVSMCs in culture to study the mechanism by which PAF stimulates proliferation of PVSMCs. We studied the effect of cell hypoxia to mimic the foetal hypoxic lung environment *in utero*, on PAF effects on cell proliferation. We also examined effects of PAF and hypoxia on expression of pertinent cytosolic and nuclear mitogenic proteins shown in our scheme of study for the hypothesis, which may be indicative of PAF receptor-mediated mitogenesis.

MATERIALS AND METHODS

Materials

Pregnant ewes (146–148-day gestation, term being 150 days) were purchased from Nebekar Farms (Santa Monica, CA, USA). Authentic standards of PAF (C₁₆-PAF) and lyso-C₁₆-PAF

Scheme 1. The following scheme illustrates our research hypothesis. PAF binding to its heptahelical membrane receptors (PAFr) activates the PAF receptor by phosphorylation. The phosphorylated PAF receptor (PAFr-P) induces phosphorylation of an extracellular signal-regulated kinase (Erk1/2) thereby activating it. Activated PAFr can also transactivate EGF receptor that can also lead to activation of Erk1/2 protein. Phosphorylated Erk1/2 in turn can activate NF-κB and induce its translocation into the nucleus. Phosphorylated Erk1/2 can also induce nuclear translocation of NF-κB through activation of other intracellular signalling proteins such as PI3 kinase. In its nuclear domain, NF-κB stimulates sequentially, the activity of the nuclear cell-cycle regulators CDK2 and CDK4 and retinoblastoma (Rb) protein. Phosphorylated Rb protein induces gene expression and cell growth.

standards, and NF-κB inhibitory peptide, PD98059, SB203580 and CV-3988 were purchased from Biomol (Plymouth Meeting, PA, USA). WEB2170 was a generous gift from Boehringer-Ingelheim (Bracknell, UK). Radiolabelled substrate, thymidine, [methyl-³H]-thymidine, 20 Ci/mmol (740.0 GBq/mmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA, USA). Phenylmethanesulphonyl fluoride (PMSF), leupeptin, pepstatin, platelet-derived growth factor (PDGF) and bovine serum albumin were purchased from the Sigma Chemical Company (St. Louis, MO, USA). The antibody to PAF receptor protein was purchased from Cayman Chemical Company (Ann Arbor, MI, USA) and Ecolite(+) liquid scintillation cocktail was purchased from MP Biochemicals (Irvine, CA, USA). Studies were always performed with freshly made up reagents.

Preparation of PVSMCs

Intrapulmonary vessels were isolated from freshly killed term foetal lambs and then SMCs were harvested from the freshly excised arteries and veins under sterile conditions, as we have previously reported (Toga *et al.* 1992). Cells were used at the 4th to 7th passage and identity of the SMCs at each passage was characterized with a SMC-specific monoclonal antibody, α-SMA (Sigma Chemical Company). The SMCs were devoid of endothelial cells and fibroblasts. Cell phenotype did not change from 4th to 7th passages as determined by the expression of α-smooth muscle actin and myosin light-chain kinase proteins.

Study conditions

All studies were performed with adherent cells either in normoxia or in hypoxia.

Normoxia

Cells were studied in a humidified incubator at 37 °C aerated with 5% CO₂ in air and oxygen concentration was monitored with a TED 60T percent oxygen sensor (Teledyne Analytical Instruments, City of Industry, CA). Incubator oxygen concentration was 21% and pO₂ as determined on the Nova Stat Profile 3 blood-gas instrument (Nova Biomedical, Waltham, MA, USA) (Ibe *et al.* 2005) was normoxic at 80–100 torr.

Hypoxia

An incubator set at 37 °C was first equilibrated for at least 1 h with a gas mixture of 2% O₂, 10% CO₂ and balance N₂ to maintain incubator pO₂ < 40 torr in the cell culture media also determined on the Nova Stat Profile 3 blood-gas instrument (Nova Biomedical) was < 40 torr (Ibe *et al.* 2005). Cells were then placed in this incubator for experimentation and the cells were continuously aerated with the hypoxia gas mixture throughout the duration of the study.

Cell culture

Pulmonary vascular smooth muscle cells from arteries (SMC-PA) and from veins (SMC-PV) were cultured in a humidified incubator aerated with 5% CO₂ in air and maintained in culture with Dulbecco's modified Eagle's medium (DMEM) containing 1% antibiotics plus an antimycotic mixture of 0.15% each of nystatin and gentamicin. Cells were subcultured at subconfluence and were used for experiments on 4–7th days of subculture. Identity of SMCs was confirmed by presence of α -smooth muscle actin antigen; cell viability was authenticated by expression of lactate dehydrogenase. Cell viability was greater than 95% from passage to passage.

Proliferation assay

Cells were seeded in 6-well culture plates at 5×10^4 cells per well and allowed to stabilize for 2–3 days. Cells were then starved by culturing in 0.1% foetal bovine serum (FBS) for 72 h. After 72 h in starving media, cells were treated with test reagents in the presence of 5 μ Ci/well of [³H]-thymidine and were incubated for 24 h more in normoxia or hypoxia, with or without inhibitors, according to the specific protocol. Test reagents were dissolved in 0.1% FBS in DMEM starving media. In experiments involving use of inhibitors, a dose–response study was performed with each inhibitor, from which optimum concentrations were selected for subsequent studies.

Assay standardization

Cells at 40–50% confluence were starved for 72 h by culturing in 0.1% FBS. Then, the starving medium was aspirated and replaced with 10% FBS growth media and the cells incubated for 24, 48 and 72 h in normoxia only. After the designated time in culture, unincorporated radioactivity was aspirated into scintillation vials and the cells were washed with calcium- and magnesium-free phosphate-buffered saline (PBS), followed by washes with 5% trichloroacetic acid. The aspirate and the washes were combined and used to determine percent incorporation of [³H]-thymidine into cells. Washed cells were detached with trypsin/ethylenediaminetetraacetic acid (EDTA) solution and spun at 1500 *g* to pellet the cells. Pellets were then lysed with 1 mL of 0.5 N NaOH or Nonidet P-40 (Sigma-Aldrich) and then spun at 480 *g* for 10 min to pellet the nuclear fraction. The 480-*g* supernatant was decanted and the radioactivity present in this supernatant fraction was also determined. The nuclear pellet was extracted with 1 mL PBS, and then the centrifuge vial was washed once with 1 mL of PBS. The extract and the wash were combined and transferred to a 20-mL scintillation vial, 10 mL of Ecolite scintillation cocktail (MP Biochemicals) was added to this nuclear fraction and the radioactivity was determined using a Beckman liquid scintillation spectrometer (Beckman Coulter, Fullerton, CA, USA).

Figure 1. Effect of different concentrations of PAF on proliferation of SMC-PA and SMC-PV. Data are means $\pm$ SEM, $n = 4$, and compared to cell proliferation produced by 10% FBS alone (control) on the cell type. In both SMC-PA and SMC-PV, PAF stimulation of cell proliferation was PAF concentration-dependent. Statistics: $*P < 0.05$, control levels and preceding PAF concentration.

From the assay standardization studies, we found that after 24 h in culture, [^3H]-thymidine incorporation into the nuclear fraction of cells (d.p.m., means $\pm$ SEM, $n = 4$) was $4.87 \times 10^5 \pm 0.036 \times 10^5$, which increased significantly by 25% to $6.37 \times 10^5 \pm 0.022 \times 10^5$ by 48 h in culture. There was no difference in cell nuclear thymidine radioactivity of $6.26 \times 10^5 \pm 0.15 \times 10^5$ by 72 h in culture compared to the 48 h incubation. The cells were already confluent by 72 h in culture. We elected to do all our subsequent studies by incubating the cells for 24 h after the 72-h starvation with 0.1% FBS and assay with 5% trichloroacetic acid and 0.5 N sodium hydroxide.

The assay was also standardized in normoxic conditions for SMC-PA and SMC-PV with respect to PAF concentrations. Studies were performed as described above using different concentrations of PAF (nM: 0.0, 1.0, 10.0 and 100.0) for 24 h and PAF-induced proliferation of SMC-PA and SMC-PV was PAF concentration-dependent. Results of these studies are shown in Fig. 1. Based on this finding of the concentration effect of PAF on cell growth, we elected to conduct all subsequent studies with 10 nM PAF and 24 h in culture.

Specific protocols

Effect of serum concentration

Effect of 0.1% serum on cell proliferation was compared to the effect of 10% serum. Furthermore, the effect of PAF stimulation of cell proliferation was studied in 0.1% and 10% FBS.

Effect of lyso-PAF and PAF receptor antagonists on cell growth

The effects of inactive PAF metabolite lyso-PAF and PAF receptor antagonist WEB2170 on cell proliferation were tested in the presence and in the absence of PAF. Serum-starved cells were pre-incubated for 2 h with 10 nM lyso-PAF, 10 μM of WEB2170, 1 μM CV-3988 or with 10% FBS growth media alone, and then 10 nM PAF and [^3H]-thymidine was added to the cells and incubated for 24 h more. The 10% FBS control received neither the WEB2170 nor the 10 nM lyso-PAF.

Effect of PDGF on cell proliferation

To show that the PAF receptor antagonist WEB2170 does not interact with other smooth muscle cell-proliferative stimuli, starved cells were pre-incubated for 2 h with 10% FBS, 10 nM PAF, 50 ng/mL PDGF (Sigma-Aldrich) or 10 μ M WEB2170. Fifty nanograms per mL PDGF was added to the cells that were pre-incubated with the WEB2170; then [3 H]-thymidine was added to all groups and incubation continued for 24 h more in normoxia only.

Role of intracellular cell-cycle regulators on PAF-induced cell proliferation

Effect of hypoxia and PAF on phosphorylation of Erk 1/2

Mitogen-activated protein kinases (MAPK) are involved in numerous cell processes, including cell proliferation and differentiation (Brunet *et al.* 1999). They are activated by extracellular signal stimuli such as hormones and cytokines *via* phosphorylation of threonine-202 and tyrosine-204 of MAPK (Erk1) or 183 and 185 (Erk2) designated as p44/p42 MAPK (Lopez-Illasaca *et al.* 1997). We previously showed that PAF activates p44/p42 in SMC-PV studied in normoxia; the effect of hypoxia and PAF on phosphorylation of p44/p42 in SMC-PV has not been reported. Here, we studied the effect of hypoxia and PAF on activation of p44/p42 in an attempt to determine involvement of this mitogenic protein on activation of downstream mitogenic proteins. Subconfluent cells were incubated for 5 h in DMEM without serum. Cells were then pulsed with 32 P-orthophosphate for 90 min in phosphate-free DMEM (MP Biochemicals). After 90 min, unincorporated radioactivity was washed off and then the washed cells were incubated for 5 min with or without 10 nM PAF in normoxia and hypoxia. After 5 min incubation, cells were subjected to immunoprecipitation and were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by phosphoimaging for phosphorylated and non-phosphorylated p44/p42. The effect of PAF on p38 MAPK protein expression was also investigated. Cells were incubated for 4 h in normoxia or hypoxia with or without 10 nM PAF, and then proteins were prepared and assayed for p38 protein expression by Western blotting.

Effect of hypoxia and PAF on NF- κ B (p65, RelA) protein [NF- κ B] expression

The effect of 15 min exposure to hypoxia and PAF on NF- κ B protein expression was also studied on SMC-PV. Cells were prepared and incubated for 15 min as described for Erk1/2 study above. The effect of PAF and hypoxia on SMC-PA and SMC-PV was also studied by incubating the cells for 24 h. For this 24-h incubation, cells were serum-starved for 72 h in normoxia with 0.1% FBS in DMEM and then incubated for 24 h with and without 10 nM PAF in normoxia and hypoxia. Cells were prepared for assay of NF- κ B protein expression by Western blotting.

To study the effect of hypoxia on nuclear translocation of NF- κ B in SMC-PA and SMC-PV, cells were grown on 150 mm Petri dishes and proteins were prepared from cells exposed to hypoxia for 24 h. Then, the proteins were subjected to differential centrifugation to prepare the nuclear fraction as follows. After 24 h incubation in normoxia or hypoxia, media were aspirated and cells were washed with ice-cold PBS. Cells were detached with trypsin-EDTA solution containing a cocktail of protease inhibitors as contained in the hypotonic lysis buffer used to prepare proteins for Western blotting. Trypsinized cells were spun on a bench centrifuge to pellet the cells. The cell pellet was washed with ice-cold PBS and spun again to re-pellet the cells. The cell pellet was suspended in the hypertonic lysis buffer, incubated on ice for 15 min and then sonicated on ice for 1 min in 15 second bouts. The lysed cells were centrifuged at 500 *g* for 10 min to harvest the nuclear fraction and the 500 *g* supernatant was centrifuged at 100 000 *g* for 1 h to harvest the cytosol. Nuclear and cytosolic localization of NF- κ B protein was assayed by Western blotting.

To define involvement of NF- κ B in PAF-induced cell proliferation, studies were performed with the NF- κ B inhibitory peptide; AAVALLPAVLLALLAPVQRKRQKLMP (Biomol) containing the nuclear localization sequence (amino acid residues 360–369) of NF- κ B p65 and the control peptide. This peptide has been shown to inhibit nuclear translocation of NF- κ B gene expression (Nagy *et al.* 2007). SMC-PA and SMC-PV were serum-starved for 72 h with 0.1% FBS cells, pulsed with 10 nM PAF for 1 h and then incubated with 50 μ g/mL of the peptide for 24 h more in normoxia or hypoxia, together with tritiated thymidine as described in general methods for assay of cell proliferation. The inhibitory peptide was titrated for concentration effect (0.1–100 μ g/mL), from which 50 μ g/mL was chosen for use. The inhibitory peptide was dissolved in 0.1% FBS.

Effect of PAF on CDK2 and CDK4 protein expression

Platelet-activating factor stimulation of CDK2 and CDK4 protein expression in SMC-PA and SMC-PV was studied in normoxia only. Serum-starved cells were incubated in normoxia with or without PAF and then proteins were prepared for measurement of CDK2 and CDK4 protein expression. All test reagents and the [3 H]-thymidine (5 μ Ci/well) were dissolved in 0.1% FBS starving medium and subsequently were added to the cells. For each protocol, cell numbers were determined by haemocytometer counting; studies were conducted as described above except that no [3 H]-thymidine was added.

Western blotting for cytosolic proteins

Protein preparation

Proteins were prepared from treated and untreated cells studied, according to the specific protocol. The lysis buffer was hypotonic containing 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Ali *et al.* 1994; Ibe *et al.* 2005), pH 7.4, together with the following cocktail of protease inhibitors; 1 mM ethyleneglycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 4 mM ethylenediaminetetraacetic acid (EDTA), 2 mM MgCl₂, 10 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulphonyl fluoride, 5 μ g/mL leupeptin, 1 μ g/mL pepstatin, 1 μ M 4-(2-aminoethyl)-benzenesulphonyl fluoride, 200 mM sodium fluoride, 20 mM sodium pyrophosphate, 0.2 mM sodium orthovanadate and 0.1 mg/mL trypsin inhibitor. Proteins were subjected to Coomassie blue quantification before Western blotting.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Studies were performed to determine optimum conditions for electrophoresis of the proteins of interest. Each protein was suspended in 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% β -mercaptoethanol, and then electrophoresed for 1 h at 200 V on 4–12% Tris-glycine gradient gels (BioWhittaker Molecular Applications, Rockland, ME, USA), along with Bio-Rad kaleidoscope prestained molecular weight markers and protein standards. After 1 h of SDS/PAGE, proteins were transferred to nitrocellulose membranes by means of Mini Trans-Blot (Bio-Rad, Redmond, CA, USA) at 70 V for 35 min. Blots were blocked with 5% non-fat dry milk in 1% Tween-20/TBS (T-TBS) overnight. Blots were then incubated for 1 h with appropriate dilution of the specific antibody against: PAF receptor protein, p38 MAPK protein, NF- κ B, CDK2 and CDK4, and retinoblastoma (Rb) proteins, after which the gels were washed with 1% T-TBS, incubated for 1 h with an antirabbit IgG HRP-linked secondary antibody (Amersham Pharmacia, Arlington Heights, IL, USA), and finally washed with 1% T-TBS. The signals were developed for 1 min using Amersham ECL Western blot detection kit and then were exposed to

radiographic film. Bands corresponding to the proteins of interest were scanned with EagleEye densitometer (Stratagene, San Diego, CA, USA) to quantify blot density. Then, blots were stripped and re-probed for total actin or glyceraldehyde 3-phosphate dehydrogenase expression, constitutively expressed proteins used as internal standards.

Data analysis

[³H]-Thymidine radioactivity obtained from each study protocol and cell numbers as determined by haemocytometer counting was normalized to the percentage of its own 10% FBS control and presented as cell number (% FBS response), as means $\pm$ SEM. All numeric data were analysed statistically by two-tail Student's *t*-test. For multiple comparisons, data were subjected to ANOVA statistics followed by the Turkey's test. Differences were considered significant at $P < 0.05$.

RESULTS

Proliferation of SMC-PA and SMC-PV is PAF concentration-dependent

Figure 1 illustrates effects of different PAF concentrations on proliferation of SMC-PA and SMC-PV, studied during normoxia. In SMC-PA, 1 nM PAF caused 23% increase in cell proliferation compared to the effect of 10% FBS alone. Proliferation increased by a further 25% with 10 nM PAF and by yet a further 21% with 100 nM PAF. For SMC-PV, 1 nM PAF caused a 52% increase in cell proliferation compared to 10% FBS alone. Proliferation increased by a further 56% with 10 nM PAF and 44% with 100 nM PAF. For both SMC-PA and SMC-PV, PAF produced concentration-dependent proliferation of the cells. Generally, the effect of PAF on SMC-PV was more than its effect on SMC-PA.

Figure 2 shows the effect of 0.1% FBS and 10% FBS on PAF stimulation of cell proliferation during normoxia and hypoxia. For SMC-PA (upper panel), during normoxia 10 nM PAF in 0.1% FBS did not produce any difference in cell proliferation compared to 0.1% FBS alone. On the other hand, 10% FBS alone produced 5-fold increase in cell population growth compared to 0.1% FBS alone. Addition of 10 nM PAF in 10% FBS produced seven times greater cell proliferation compared to the effect of 0.1% FBS alone or the effect of 10 nM PAF in 0.1% FBS. For SMC-PA, hypoxia alone produced 80% increase in cell proliferation compared to 0.1% FBS in normoxia. Addition of 10 nM PAF in 0.1% FBS did not produce any change in cell growth compared to 0.1% FBS alone in hypoxia. During hypoxia, 10% FBS produced 4-fold greater cell growth than 0.1% FBS alone in hypoxia, over 2-fold increase in cell proliferation compared to the effect of 10% FBS alone in normoxia, and 46% greater cell proliferation than 10% FBS alone in hypoxia. In addition, 10 nM PAF in 10% FBS produced 56% increase in cell proliferation compared to the effect of 10 nM PAF in 10% FBS during normoxia.

In SMC-PV, during normoxia (lower panel), 10 nM PAF in 0.1% media did not increase cell population growth compared to 0.1% FBS alone. On the other hand, 10% FBS produced 3-fold increase in cell proliferation compared to 0.1% FBS alone in normoxia or 10 nM PAF in 0.1% FBS in normoxia. Addition of 10 nM PAF to 10% FBS in normoxia produced 23% increase in cell proliferation compared to 10% FBS alone, and a 4-fold increase in cell proliferation compared to either 0.1% FBS alone or 10 nM PAF in 0.1% FBS. During hypoxia, 0.1% FBS produced no change in cell proliferation compared to 0.1% FBS alone in normoxia. Addition of 10 nM PAF to 0.1% FBS in hypoxia also produced no significant change in cell proliferation compared to the effect of 10 nM PAF in normoxia or the effect of 0.1% FBS alone during normoxia and hypoxia. On the other hand, 10% FBS alone produced 60% greater cell proliferation

Figure 2. Comparison of effect, 0.1% FBS, 10% FBS and 10 nM PAF of proliferation. SMC-PA is in the upper panel and SMC-PV in the lower panel. Data are means $\pm$ SEM, $n = 6$. Cell proliferation was studied by [³H]-thymidine incorporation as described in the Materials and Methods section. Ten nanomolars PAF in 0.1% FBS did not stimulate cell growth compared to 0.1% FBS alone. 10% FBS stimulated cell growth compared to 0.1% FBS and 10 nM PAF in 10% FBS further stimulated cell growth. Proliferation of SMC-PV is greater than proliferation of SMC-PA under all the conditions. Statistics: * $P < 0.05$, normoxia; ** $P < 0.05$, 0.1% FBS conditions.

than 10% FBS alone in normoxia, which is 5-fold greater cell proliferation compared to effect of 0.1% FBS alone, and effect of 10 nM PAF in 0.1% in normoxia and hypoxia. Addition of 10 nM PAF to 10% FBS resulted in 54% increase in cell proliferation compared to effect of 10 nM PAF in 10% FBS during normoxia; 19% increase in cell proliferation compared to 10% FBS alone in hypoxia; 57% increase compared to 10% FBS in normoxia; and 7-fold increase compared to the effect of 0.1% FBS alone or 10 nM PAF in 0.1% FBS in normoxia and hypoxia. In general, during normoxia, proliferation of SMC-PV in 0.1% FBS was 5-fold greater than proliferation of SMC-PA and in 10% FBS, SMC-PV proliferation was 3-fold greater than proliferation of SMC-PA. The corresponding values for greater proliferation of SMC-PV during hypoxia compared to

Figure 3. Baseline proliferation of SMC-PA and SMC-PV determined by haemocytometer counting. Data are means $\pm$ SEM, $n = 4$. Cell proliferation was studied as described in the Materials and Methods section without [^3H]-thymidine. Hypoxia stimulated cell proliferation in SMC-PA and SMC-PV. Proliferation of SMC-PV was greater than proliferation of SMC-PA. Statistics: * $P < 0.05$, normoxia; ** $P < 0.05$, SMC-PA.

SMC-PA are: 2.5-fold in 0.1% FBS, and 3.5-fold in 10% FBS. Furthermore, compared to SMC-PA, the effect of 10 nM PAF on SMC-PV proliferation was 2-fold greater in normoxia and hypoxia.

Because 10% FBS produced a greater effect on cell proliferation than 0.1% FBS, we studied cell proliferation by cell counting, using a haemocytometer. The result is shown in Fig. 3 as cell number, means $\pm$ SEM ($\times 10^3$ cells/well). In SMC-PA during normoxia, cell number was 8.3 ± 2.2 . Hypoxia increased cell number by 38%, indicating active cell proliferation in 10% FBS. In SMC-PV, cell number during normoxia was 30.2 ± 4.5 , which is 3.6-fold more than growth of SMC-PA in normoxia. Hypoxia caused a 50% increase in cell population growth compared to cell population growth in normoxia, also indicating active proliferation of the SMC-PV in hypoxic conditions. In general, SMC-PV showed 2- to 3-fold greater cell proliferation than SMC-PA in normoxia and hypoxia, respectively. Thus, whether by [^3H]-thymidine incorporation or by cell counting, hypoxia augmented cell proliferation and then SMC-PV showed greater cell proliferation than SMC-PA.

The inactive PAF analogue, lyso-PAF did not stimulate cell proliferation and PAF receptor antagonist WEB2170 inhibited PAF-induced cell proliferation

Figure 4 shows the effect of lyso-PAF and WEB2170 on proliferation of intrapulmonary SMCs. For SMC-PA during normoxia, treatment of cells with 10 nM PAF increased cell proliferation by 50%. Treatment of cells with 10 nM lyso-PAF neither increased nor decreased cell proliferation produced by 10% FBS alone. On the other hand, when the cells were treated with WEB2170 either alone or in combination with PAF, cell proliferation was inhibited by 45% and WEB2170 inhibition of cell proliferation was not reversed by PAF. During hypoxia, cell proliferation increased by 40% compared to 10% FBS in normoxia. Treatment of cell with lyso-PAF alone produced no change compared to effect of 10% FBS alone under hypoxia. On the other hand, treatment of cells with WEB2170 alone or in combination with PAF resulted in 70% inhibition of cell proliferation. There was no difference in the effect of WEB2170 on cell proliferation during normoxia and hypoxia.

Figure 4. Comparison of effect of PAF, lyso-PAF, WEB2170, and CV-3988 on cell growth. SMC-PA in the upper pane and SMC-PV in the lower panel in 10% FBS. Data are means $\pm$ SEM, $n = 6$. Cell proliferation was studied by [3 H]-thymidine incorporation as described in the Materials and Methods section. 10 nM lyso-PAF had no effect on cell growth while 10 μ M WEB 2170 attenuated cell growth. Statistics: * $P < 0.05$, normoxia; ** $P < 0.05$, PAF and 10% FBS conditions; # $P < 0.05$, 10% FBS in normoxia and hypoxia.

In SMC-PV during normoxia, 10 nM PAF stimulated cell proliferation and under hypoxia, and then hypoxia enhanced cell proliferation either in the absence or presence of 10 nM PAF. Treatment of cells with lyso-PAF did not alter the profile of cell proliferation compared to 10% FBS alone. Thus, similar to observations with SMC-PA, lyso-PAF neither inhibited nor stimulated proliferation of SMC-PV. However, treatment of the cells with WEB2170 resulted in significant inhibition of PAF-stimulated cell proliferation during normoxia and hypoxia. As was observed in SMC-PA, there was no difference in effect of WEB2170 on PAF-stimulated cell proliferation during normoxia and hypoxia. PAF receptor-mediated cell proliferation in SMC-PA and SMC-PV was also inhibited by the specific PAF receptor antagonist CV-3988.

To test the specificity of WEB2170 in inhibiting PAF receptor-mediated cell proliferation, we tested its effect on cell proliferation induced by PDGF, which is another proliferative agent. We performed this study on SMC-PA during normoxia only. Compared to the effect of 10% FBS growth media (100% proliferation), 50 ng/mL PDGF treatment produced $185 \pm 3.5\%$ cell growth, that is 85% greater effect than effect of 10% FBS alone. When cells were pulsed with 10 μM WEB2170 for 2 h and then treated with 50 ng/mL PDGF, cell proliferation was $193 \pm 10.7\%$, a non-significant change compared to the effect of PDGF alone. This indicates that WEB2170 does not inhibit cell proliferation caused by another agent acting *via* a non-PAF receptor-mediated pathway.

Hypoxia plus PAF increases phosphorylation of MAPK subtype Erk1/2 (p44/42) proteins

Because proliferation of SMC-PV was over 2-fold greater than proliferation of SMC-PA, we studied the effect of short time-period exposure of cells to hypoxia plus PAF, on phosphorylation of Erk1/2. Figure 5a shows the effect of 5 min incubation on phosphorylation of Erk1/2 measured as ^{32}P radioactivity. Addition of 10 nM PAF to cells in normoxia produced a 4-fold increase in ^{32}P radioactivity in the Erk1/2 band, indicating greater phosphorylation of the kinases. Incubation of the cells in hypoxia under baseline conditions, produced over 3-fold increase in Erk1/2 phosphorylation compared to baseline conditions in normoxia. Addition of 10 nM PAF to cells in hypoxia led to a 2-fold increase in phosphorylation compared to baseline conditions in hypoxia and 6-fold increase in phosphorylation compared to baseline conditions in normoxia. PAF treatment produced a 55% increase in Erk1/2 phosphorylation compared to phosphorylation of PAF-treated cells in normoxia. Thus, 5 min hypoxia augments Erk1/2 phosphorylation and treatment with 10 nM PAF for 5 min further increases phosphorylation over hypoxia alone.

Hypoxia and PAF stimulate MAPK p38 protein expression

The effect of PAF and hypoxia on expression of p38 subtype of MAPK by SMC-PA is shown in Fig. 5b, and by SMC-PV is shown in Fig. 5c. SMC-PA treated with 10 nM PAF in normoxia, expressed greater p38 protein by 45%. Incubation of cells in hypoxia alone increased p38 protein expression to a level comparable to the effect of PAF under normoxia. Treatment of cells with PAF in hypoxia significantly increased p38 protein by 25% compared to the effect of hypoxia alone. Thus, the condition of hypoxia plus PAF increases p38 protein expression in SMC-PA. With respect to SMC-PV (Fig. 5c), treatment of cells with PAF in normoxia increased

Figure 5. (a) Representative phosphoimages (left panel) and phosphoimage analysis (right panel). The effects of PAF stimulation of Erk1/2 phosphorylation (p44/p42) in normoxia and hypoxia. Data are means $\pm$ SEM, $n = 4$. SMC-PV was pulsed with ^{32}P orthophosphate in normoxia for 90 min and then stimulated with hypoxia and PAF for 5 min. Phosphorylated Erk1/2 was measured as described in the Materials and Methods section. Hypoxia stimulated phosphorylation Erk1/2 (phospho-p44 MAPK, phospho-p42 MAPK) and treatment with 10 nM PAF augmented the phosphorylation. Statistics: $*P < 0.05$, control conditions/normoxia; $**P < 0.05$, control conditions/normoxia and hypoxia. (b,c) Representative Western blot and densitometry analyses of effects of PAF and hypoxia. Stimulation of p38 MAPK protein expression in SMC-PA (Fig. 5b) and SMC-PV (Fig. 5c). Numerical data are means $\pm$ SEM, $n = 6$. Studies were conducted as described in the Materials and Methods section. Labels are: lanes 1 and 3, control conditions in normoxia and hypoxia, respectively; lanes 2 and 4, protein expression with PAF treatment in normoxia and hypoxia, respectively. In SMC-PA, PAF and hypoxia stimulated p38 protein expression by the cells, but in SMC-PV hypoxia did not augment PAF stimulation of p38 protein expression. Statistics: $*P < 0.05$, control condition in normoxia or hypoxia; $\dagger P < 0.05$, normoxic conditions. (d) Effect of the MAPK inhibitors, PD98059 and SB203508. PAF induced SMC-PA and SMC-PV proliferation. Data are means $\pm$ SEM, $n = 6$ and are presented as percentage of proliferation caused by the control, which is 10% FBS growth media. Both inhibitors, PD98059 (PAF+PD) and SB203508 (PAF+SB), attenuated PAF-induced cell proliferation during normoxia and hypoxia. Statistics: $*P < 0.05$, PAF treatment alone in normoxia or hypoxia.

p38 protein expression by 80%. Incubation of cells under hypoxia alone did not produce any change in p38 protein expression compared to control levels under normoxia. However, treatment of cells with PAF under hypoxia significantly increased p38 protein expression by 75% compared to the effect of hypoxia alone. Unlike in SMC-PA, hypoxia alone did not influence p38 protein expression.

Platelet-activating factor-induced cell proliferation was inhibited by MAPK inhibitors

Figure 5d shows the effect of the MAPK inhibitors PD98059 and SB203508, on PAF-induced cell proliferation in normoxia and hypoxia. The purpose of the experiment was neither to compare the inhibitory potency of the compounds on each cell type nor to assess any differences in inhibition in normoxia and hypoxia. The results show that both PD98059 and SB203508 inhibited PAF-induced cell proliferation.

Hypoxia and PAF exposure increase NF- κ B protein expression by SMC-PV and SMC-PA

Figure 6a shows NF- κ B protein expression following 15 min treatment of SMC-PV during normoxia and hypoxia, and quantified as 32 P radioactivity determined on a phosphoimager. Incubation of SMC-PV for 15 min in normoxia induced NF- κ B protein expression and detectable radioactivity (d.p.m., means $\pm$ SEM). Incubation of the cells in normoxia without added PAF (control) induced NF- κ B protein expression and 0.91 ± 0.10 32 P radioactivity. Incubation of cells in hypoxia without added PAF also induced NF- κ B expression and a significant increase in 32 P radioactivity, 2.51 ± 0.12 , although the blots look similar visually. Incubation of cells in normoxia with 10 nM PAF led to a 3-fold increase in 32 P radioactivity, 3.17 ± 0.15 , and in hypoxia with 10 nM PAF, the increase was 2-fold and 5.67 ± 0.31 in 32 P radioactivity, and as in the control conditions, the size of the blots are visually similar. Thus, 15 min exposure of cells to hypoxia increases NF- κ B phosphorylation and treatment of cells with PAF in normoxia, and hypoxia increases NF- κ B protein expression and enhances phosphorylation. This result suggests that whereas hypoxia alone may not induce greater NF- κ B expression within 15 min, hypoxia does induce phosphorylation of the protein.

Figure 6b shows the effect of 24 h hypoxia and 10 nM PAF on NF- κ B expression. In SMC-PA (Fig. 6b), NF- κ B protein was expressed after 24 h in culture in normoxia. Incubation with 10 nM PAF increased protein expression by 40% over control conditions. Incubation in hypoxia alone for 24 h increased protein expression 32% over control conditions in normoxia. Addition of 10 nM PAF under hypoxia led to 18% increase in protein expression over control conditions in hypoxia, 12% increase over protein expression in presence of PAF in normoxia and 55% increase over control conditions in normoxia. In SMC-PV (Fig. 6c), 24 h incubation in normoxia also led to NF- κ B protein expression. Addition of 10 nM PAF in normoxia increased protein expression by 26% over control conditions normoxia. Incubation in hypoxia alone for 24 h increased protein expression by 30% over control conditions in normoxia. Addition of 10 nM PAF in hypoxia increased protein expression by 30% over control conditions in hypoxia, 33% over effect of 10 nM PAF in normoxia and 67% over protein expression in control conditions in normoxia. Our studies were not designed to quantify differences in NF- κ B protein expression in both cell types. However, in both cell types, hypoxia increased NF- κ B protein expression and addition of 10 nM PAF further increased protein expression.

Platelet-activating factor induces greater expression of NF- κ B protein in the nuclear fraction of SMC-PA and SMC-PV

Figure 7a and b illustrates the effects of PAF on NF- κ B protein expression in the nuclear fraction and cytosol of SMC-PA and SMC-PV cultured under hypoxia with and without 10 nM PAF

Figure 6. (a) Representative Western blot of phosphorylated NF-κB protein following 15 min incubation in normoxia and hypoxia. The ^{32}P radioactivity was determined on phosphoimager. Numerical data are means $\pm$ SEM, $n = 3$ and described in text of Fig. 6a in the Results section. The labels are: lanes 1 and 2 control conditions in normoxia and hypoxia, respectively; lanes 3 and 4 PAF treatment in normoxia and hypoxia, respectively. PAF stimulated expression of NF-κB and phosphorylation of NF-κB protein was augmented by hypoxic conditions. (b,c) Representative Western blot (upper panel) and densitometry (lower panel) analyses. PAF and hypoxia effect on NF-κB protein expression in SMC-PA (Fig. 6b) and SMC-PV (Fig. 6c). Numerical data are means $\pm$ SEM, $n = 6$. Studies were conducted as described in the Materials and Methods section. Labels are: lanes 1 and 3, in control conditions in normoxia and hypoxia, respectively; lanes 2 and 4, protein expression with PAF treatment in normoxia and hypoxia, respectively. Hypoxia increased NF-κB protein expression and treatment with 10 nM PAF further increased expression in normoxia and hypoxia. Statistics: * $P < 0.05$, control; † $P < 0.05$, control and PAF treatment in normoxia.

treatment; NF-κB protein was expressed in the nuclear and cytosolic fractions of both cell types. In SMC-PA (Fig. 7a), PAF induced 30% greater NF-κB protein expression in the nucleus than without PAF treatment. Furthermore, expression in the cytosol was more than 4-fold greater with PAF treatment. With SMC-PV (Fig. 7b), PAF-induced nuclear expression was 7-fold greater than without PAF treatment. In addition, PAF induced greater expression of NF-κB protein in the cytosol.

Figure 7. (a,b) Representative Western blot (upper panel) and densitometry analyses. Effects of PAF and hypoxia on stimulation of cytosolic and nuclear expression of NF- κ B protein in SMC-PA (Fig. 7a) and SMC-PV (Fig. 7b). Numerical data are means $\pm$ SEM, $n = 4$. Studies were conducted as described in the Materials and Methods section. PAF stimulated nuclear translocation of NF- κ B protein during hypoxia in both cell types. Labels are as follows: lanes 1 and 3, nuclear NF- κ B protein expression in the absence and presence of 10 nM PAF, respectively, in SMC-PA and SMC-PV; lanes 2 and 4, cytosolic NF- κ B protein expression in the absence and presence of PAF, respectively, in SMC-PA and SMC-PV. PAF stimulated nuclear expression of NF- κ B by both cell types. Statistics: * $P < 0.05$, nuclear expression; † $P < 0.05$, condition without PAF treatment. (c) Effect of NF- κ B inhibitory peptide on PAF stimulation of SMC-PA and SMC-PV proliferation. Data are means $\pm$ SEM, $n = 4$. Serum-starved cells incubated for 24 h with 50 μ g/mL of the inhibitory peptide, and cell proliferation, and cell proliferation, were studied as described in the Materials and Methods section. NF- κ B inhibitory peptide attenuated PAF-induced cell proliferation. Statistics: * $P < 0.05$, PAF treatment alone.

Nuclear factor-kappa B inhibitory peptide attenuates PAF-stimulated pulmonary vascular smooth muscle cell proliferation

Nuclear factor-kappa B inhibitory peptide has 26-amino acid residues and it inhibits NF- κ B-mediated cell-cycle responses. In Fig. 7c, treatment of cells with 10 nM PAF in the presence of the 50 μ g/mL of the peptide produced significant inhibition of cell proliferation in both

Figure 8. Representative Western blot and densitometry. Western blot (upper panel) and densitometry (lower panel) of the effect of PAF and hypoxia on stimulation of CDK2 and CDK4 protein expression in SMC-PA (Fig. 8a) and SMC-PV (Fig. 8b). Numerical data are means $\pm$ SEM, $n = 6$. Studies were conducted as described in the Materials and Methods section. Labels are: lanes 1 and 3, baseline studies in SMC-PA and SMC-PV, respectively; lanes 2 and 4, protein expression after PAF treatment in SMC-PA and SMC-PV, respectively. PAF treatment enhanced protein expression by each cell type and then expression of each protein with or without PAF was greater in SMC-PV. Statistics: * $P < 0.05$, baseline conditions; † $P < 0.05$, SMC-PA values.

SMC-PA and SMC-PV during normoxia and hypoxia, compared to the effect of 10 nM PAF alone. There was no difference between inhibition of cell proliferation in normoxia or under hypoxia.

Platelet-activating factor stimulates CDK2 and CDK4 protein expression more in SMC-PV than in SMC-PA

Cyclin-dependent kinases are nuclear NF- κ B-linked cell-cycle regulators. Figure 8 shows expression of CDK2 and CDK4 proteins by cells studied during normoxia only. In SMC-PA, CDK2 expression under baseline conditions (Fig. 8a), illustrates only 0.04% of the GAPDH protein standard. Addition of 10 nM PAF produced an over 2-fold increase in expression. The corresponding values for SMC-PV (Fig. 8b) are 1.95% at baseline conditions, which increased by 35% on addition of 10 nM PAF. With respect to CDK4 protein expression in SMC-PA, CDK4 expression in baseline conditions was 0.28% of GAPDH standard protein expression that increased by over 2-fold to 0.71, with addition of 10 nM PAF. The corresponding values for SMC-PV are 1.93% under baseline conditions that increased by 23% on addition of 10 nM PAF. The Western blot was standardized for protein loading. The data suggest that expression and activity of both CDK2 and CDK4 are greater in SMC-PV than in SMC-PA, and that PAF treatment augments expression of the two kinases in both cell types.

DISCUSSION

During development and physiological growth, proliferation of PVSMCs plays an important role in normal growth of the vascular system (Carmeliet 2000). Proliferation of vascular SMCs is initiated by endogenous and exogenous stimuli, which may involve autocrine or paracrine

mechanisms (Berk 2001). Induction of cell proliferation by an agent will entail activation of specific signals controlling cell division and cell population growth. NF- κ B and cyclin-dependent kinases are intracellular regulators of cell division and cell growth (Prendes *et al.* 2003; Berthet *et al.* 2006). PAF is an endogenous lipid mediator that can activate cell division by autocrine and paracrine mechanisms (McManus & Pinckard 2000). We employed pharmacological manipulation in an *in vitro* setting, to describe a mechanism by which PAF induces proliferation of PVSVC in normoxic and hypoxic conditions. Our data show that: (i) smooth muscle cells from pulmonary veins proliferate more than cells from pulmonary arteries in normoxia and under hypoxia and that stimulation of the cells with PAF augments cell proliferation in both conditions; (ii) PAF induces proliferation of the cells *via* a PAF receptor-specific pathway; (iii) hypoxia induces phosphorylation of Erk1/2 and PAF treatment augments the phosphorylation in normoxia and hypoxia; (iv) PAF and hypoxia induce expression of MAPK p38 protein; (v) short-term (15 min) treatment of cells with PAF-induced expression of the intracellular mitogenic protein NF- κ B with significant phosphorylation measured as 32 P radioactivity; (vi) extended duration of hypoxia stimulates expression of NF- κ B and treatment of cells with PAF augmented this expression; (vii) hypoxia and PAF stimulate nuclear translocation of NF- κ B and the NF- κ B inhibitory peptide inhibited PAF-stimulated cell proliferation; (viii) PAF augments expression of the cyclin dependent kinases, CDK2 and CDK4 in both SMC-PA and SMC-PV. We further show that culture of the SMCs in 10% FBS was necessary to stimulate cell growth. This finding is in support of the inclusion of high percentage of bovine serum albumin in all *in vitro* studies involving PAF. Serum is necessary to solubilize PAF, a lipophilic molecule, and transport it into the cell. In line with our primary hypothesis, our data show that PAF stimulates ovine foetal pulmonary vascular smooth muscle cell proliferation *in vitro* and suggest that PAF stimulation of cell growth occurs *via* an NF- κ B linked pathway. Our experiments with PDGF and WEB2170 together with the effect of CV-3988 on cell growth demonstrate that the effect of PAF on these cells occurs *via* PAF receptor-mediated mechanisms.

Physiological and pathological effects of PAF are mediated by its specific G-protein-coupled receptor. PAF induces expression of its own receptors *in vivo* and *in vitro* suggesting that it activates intracellular molecules that regulate its gene expression and cell replication (Wang *et al.* 1997; Ibe *et al.* 2007). NF- κ B is a transcription factor of which the most common isoform is a heterodimer of p50 and p65 subunits, which maintain a cytoplasmic domain until activated by a stimulus. RelA NF- κ B p65 also known as RelA, CDK2 and CDK4, and Rb protein, are known intracellular regulators of cell division and cell growth, achieved by communicating distinct signals to different proteins within the cytosol (Amrani *et al.* 2003; Berthet & Kaldis 2006; Berthet *et al.* 2006). Mitogen-activated protein kinase Erk1/2 is an upstream stimulus for NF- κ B activation (Vanden Berghe *et al.* 1998). In this report, we found that hypoxia and PAF increased Erk1/2 phosphorylation and a combination of hypoxia and PAF further increased the phosphorylation. Protein phosphorylation is a rapid and reversible reaction employed in elucidation of signal transduction mechanisms (Pearson *et al.* 2001; Vermeulen *et al.* 2003). Although a single kinase in a signalling pathway can affect multiple signalling proteins and transcription factors, phosphorylation of a signalling protein is helpful in understanding the function of the proteins of interest. Furthermore, the two cell types expressed p38 protein when stimulated with PAF. PAF-induced cell proliferation was inhibited by specific inhibitors of MAPK isotypes Erk1/2 and p38, PD98059 and SB203508, showing that MAP kinases are active participants in PAF receptor-mediated PVSVC proliferation. Our data show that hypoxia and PAF-induced phosphorylation of Erk1/2 may be an initial event in the activation of downstream signals such as NF- κ B, which would subsequently lead to pulmonary smooth muscle cell proliferation.

Activation of NF- κ B can proceed by two different pathways, the canonical pathway by which different stimuli activate upstream effector proteins anchored by Ikappa kinase (IKK) isoforms, and the non-canonical pathway that requires only IKK α (Perkins 2006). No matter the preceding upstream pathway, activation of NF- κ B involves some post-translational modifications that include nuclear translocation from its cytoplasmic domain. After nuclear translocation, induction of gene expression and cell proliferation by NF- κ B is modulated by other mitogenic proteins of nuclear origin. In this study, PAF induced phosphorylation of NF- κ B within 15 min exposure, but hypoxia did not enhance this expression. However, longer exposure to hypoxia augmented NF- κ B expression over the effect PAF alone. This is different from the effect of PAF and hypoxia on Erk 1/2 phosphorylation. This suggests that PAF-induced phosphorylation of NF- κ B p65 isoform is one further form of its other post-translational modification, and that hypoxia may serve to up-regulate NF- κ B expression. Hypoxia-inducible factor-1 α (HIF-1 α) is an important regulator of vascular smooth muscle cell proliferation (Iyer *et al.* 1998). Partial HIF-1 α deficiency decreases muscularization of small pulmonary arteries of mice exposed to chronic hypoxia (Yu *et al.* 1999). Recently, Schultz associates (2006), showed that proliferation of human pulmonary artery SMCs induced by mitogens such as PDGF and EGF during hypoxia, were not modulated by HIF-1 α , but a role for HIF-1 α in PAF-induced cell proliferation has not been reported. In addition, when we examined the role of VEGF in PAF-induced cell proliferation during normoxia and hypoxia, we found that VEGF (10 ng/mL) attenuated PAF-induced SMC-PA proliferation by $23 \pm 9.05\%$ and $46.28 \pm 6.96\%$ under normoxia and hypoxia, respectively. Corresponding values for SMC-PV are; $54.0 \pm 7.55\%$ and $51.06 \pm 8.70\%$ in normoxia and hypoxia, respectively. This information together with the results of our investigation with PDGF, demonstrated that the PAF effects presented in this report do not involve the contributions by PDGF or VEGF.

Cyclin-dependent kinases are nuclear downstream effectors of NF- κ B functions such as induction of gene expression and cell growth (Pearson *et al.* 2001; Vermeulen *et al.* 2003; Perkins 2006; Suh *et al.* 2006). During growth, cells pass the restriction point in late G₁ phase to begin DNA replication (Malumbres *et al.* 2000). CDK2 and CDK4 catalyse phosphorylation of Rb in late and early G₁ phases, respectively, and then after phosphorylation of Rb, transition of the cell cycle to S phase occurs (Jacks *et al.* 1992), and cell-cycle progression is regulated by CDKs after activation by NF- κ B and its upstream effector proteins (Gao *et al.* 2004; Dutta *et al.* 2006). In this study, we found that hypoxia and PAF stimulated expression of NF- κ B protein above baseline conditions and PAF as well as hypoxia stimulated its nuclear translocation, suggesting that this nuclear translocation is the antecedent of activation of CDK2 and CDK4 proteins and subsequent stimulation of gene expression and cell proliferation. Effects of PAF and/or hypoxia were compared to expression of the proteins by cells, studied in 10% FBS culture medium, after 72 h serum starvation. This protocol was necessary as in a previous report, Gao *et al.* (2004) showed that serum is necessary for activation CDKs and Rb protein, and we found that 10% serum is required for PAF to stimulate cell proliferation. Recently, we showed that PAF stimulation of SMC-PV proliferation occurs *via* transactivation of the EGF receptor. In this report, we show that PAF also induces SMC-PA and SMC-PV proliferation by activation of NF- κ B. At present, it is not clear whether NF- κ B activation is synchronized with transactivation of the EGF receptor by PAF, and the intracellular locale of this apparent synchronization is also not yet understood. Nevertheless, our report presents new information in our quest to understand factors that regulate cell growth *in vivo* and *in vitro*. These data suggest that *in vivo*, in the hypoxic environment of foetal lungs, a combination of hypoxic conditions and PAF stimulate cell growth and enhance vascularization of the lung. When we tested for specificity of NF- κ B effect on proliferation of SMC-PA and SMC-PV, we found that the NF- κ B inhibitory

peptide significantly attenuated PAF-induced cell proliferation under both normoxia and hypoxia. Our present report supports a role for NF- κ B p65 isoform in growth of PVSMCs.

In foetal pulmonary circulation, vasoconstrictors such as PAF (Ibe *et al.* 1998) and endothelin-1 (Hassoun *et al.* 1992) are mitogenic and maintain high vasomotor tone and aid in sustaining vascular development. At birth, release of vasodilators, such as nitric oxide, by the endothelium (Palmer *et al.* 1988) induces smooth muscle relaxation (Ignarro *et al.* 2001). In ovine foetal PVSMCs, hypoxia up-regulates PAFr-binding and PAFr-mediated intracellular inositol 1,4,5-trisphosphate and calcium release (Ibe *et al.* 2005); therefore, the foetal hypoxic environment facilitates PAFr-binding, PAFr-mediated signalling and maintenance of high pulmonary vasomotor tone *in utero*. PAF may actively down-regulate vasodilator pathways in pulmonary circulation of the foetal hypoxic environment (Ibe *et al.* 2007). We have reported that in pulmonary vascular smooth muscle, PAF receptor protein expression, PAF receptor-binding and PAF receptor-mediated intracellular signalling are down-regulated by vasodilators such as cGMP/PKG and cAMP/PKA (Ibe *et al.* 2007). Thus, abnormal regulation of functions of the smooth muscle and the endothelium, such as inability to down-regulate SMC- and endothelium-derived vasoconstrictors, and up-regulation of endothelium-derived vasodilators, in the perinatal period is implicated in the pathogenesis of persistent pulmonary hypertension of the neonate (PPHN) (Fineman *et al.* 1995; Wedgwood & Black 2005; Nagy *et al.* 2007). Pulmonary arteries of newborns who die from PPHN are characterized by hyperplasia of SMCs and extension of smooth muscle to nonmuscular arteries (Haworth & Reid 1976). Thus, down-regulation of PAF-induced cell proliferation, postnatally, may be one mechanism of preventing pulmonary vascular abnormalities of PPHN.

In conclusion, our data show that hypoxia stimulates pulmonary vascular smooth muscle cell proliferation, and treatment with physiological concentrations of PAF augments the effect of hypoxia. Hypoxia and PAF stimulate phosphorylation of Erk 1/2 and activate NF- κ B and the nuclear cell-cycle regulators CDK2 and CDK4 suggesting that the PAF effect on cell replication is anchored to expression of these intracellular mitogenic proteins. We speculate that *in vivo*, in the hypoxic environment of foetal lungs where PAF levels are high, PAF acts *via* its receptor to induce proliferation of SMCs, an important role in the maintenance and remodelling of the foetal pulmonary vascular system during development and physiological growth *in utero*. We also speculate that uncontrolled cell growth, postnatally, *via* inability to down-regulate PAF receptor-mediated effects, may lead to hyperplasia of pulmonary vascular smooth muscle and incidence of persistent pulmonary hypertension of the neonate.

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