**RESEARCH ARTICLE**

# Platelet Activating Factor Receptor Binding and Contractile Protein Expression by Ovine Fetal Pulmonary Vascular Smooth Muscle Cells: Role in Rho Kinase Mediation of PAF Receptor-Linked Physiological Responses

**Authors**
Basil O. Ibe, Amy M. Douglass, Stephen M Douglass, Lissette S. Renteria

**Affiliations**
Division of Neonatology, Department of Pediatrics, The Lundquist Institute at Harbor-UCLA Medical Center, Torrance, CA 90502

**Corresponding author**
Basil O. Ibe, Ph.D.
Division of Neonatology
The Lundquist Institute
1124 West Carson Street, RB3
Torrance, CA 90502
Phone : (310) 222-1966
Fax: (310) 222-3887
E-mail: ibe@lundquist.org

**Abstract**

We studied effects of hypoxia and some modulators of PAFR–mediated signaling in fetal ovine arterial and venous smooth muscle cells to aid in explaining PAF regulation of fetal pulmonary hemodynamics *in utero*. PAFR, a G protein coupled receptor, is characteristically measured in membrane proteins. Profile of binding to soluble and nuclear fraction proteins from SMC have not been studied. Rho kinase (ROCK) inhibitors decrease PAFR-linked smooth muscle growth, suggesting that PAFR internalization may not be only the pathway to downregulate PAFR-mediated responses. We hypothesized that PAF binds to SMC subcellular protein fractions and together with ROCK contributes to physiological effects of PAF. Protein fractions were prepared from cells cultured in normoxia or hypoxia. PAF binding, PAFR protein and gene expression were studied. In normoxia, PAFR density ($B_{max}$) in arterial and venous SMC were 487±37 and 624±17 respectively, which increased by 30-35% in hypoxia, and was greater in venous than arterial SMC in normoxia and hypoxia. The $K_D$ for PAFR binding to arterial cells was higher than for venous, and then hypoxia decreased the $K_D$ of PAFR binding in both cell types. PAFR binding to subcellular fractions were detectable, but were significantly lower than for membrane fractions in all conditions; e.g., normoxia: venous versus membrane, soluble fraction, 40%; nuclear fraction, 20%. ROCK inhibitor (Y-27632) attenuated PAFR binding after 24 hr incubation comparable to effects of cycloheximide and CV-3988. Unlike CV-3988, Y-27632 had no effect on PAF binding to pre-synthesized receptors within 30 min incubation. Y-27632 and HA-1077 increased MLCK and Calponin expression, although oxygen-related effects in venous SMC were different. PAFR gene expression was higher in hypoxia and effect of PAF and CV-3988 treatment on gene expression was paradoxically higher in arterial SMC supporting a translational control of PAFR activity in lung SMC. This study shows that PAFR binding to the SMC is not exclusive to membranes and that hypoxia in conjunction with ROCK modulate PAFR-mediated signaling in pulmonary vascular SMC in vitro with different effects on cells from arteries and veins. Different effects of ROCK on contractile proteins expression in hypoxia suggest involvement of these proteins in PAF-induced lung SMC reactivity in utero.

**Key word:** PAF receptor, Protein fractions, hypoxia, Rho kinase, MLCK, Calponin

## Introduction

Platelet activating factor (PAF) is an important mediator of pulmonary circulation[1-5]. In utero, endogenous PAF maintains high pulmonary vasomotor tone and vascular resistance in fetal lambs[3]. The role of PAF in the pathogenesis of hypoxia-induced pulmonary vascular remodeling has been studied in some animal models in vivo and in vitro[6, 7, 8]. In ovine fetal lung in utero, increased PAFR protein expression and PAFR binding contribute to pulmonary vascular remodeling in these animals and may predispose them to persistent pulmonary hypertension after birth[6]. PAF acts through its specific G protein-coupled receptors (GPCR) to evoke vasoconstriction, and other physiological responses[1, 3, 9, 10, 11, 12]. PAF-induced smooth muscle contraction is mediated by Rho kinase (ROCK) resulting in $Ca^{2+}$ release and smooth muscle constriction[13]. ROCK is a down-stream target of small GTP-binding protein Rho, which regulates cell motility and $Ca^{2+}$ sensitization. Inhibition of Rho-kinase activity with Y-27632 attenuates PAF-induced

pressor responses in isolated perfused lungs[13] in support of ROCK effect in modulation of pulmonary vascular reactivity**.** Rho kinase is a member of Rho family of low molecular weight G proteins which regulate a variety of cell functions including cell growth, gene expression, $Ca^{2+}$ sensitization, and cytoskeletal rearrangement[14, 15, 16]. Rho kinase activities are regulated by extra cellular stimuli, which activate some cell surface receptors[14, 17.] Studies investigating the involvement of Rho kinase in various cellular responses have been facilitated by the generation of RhoA cDNA constructs and specific ROCK inhibitors[16, 18, 19, 20]. PAFR binding induces an intracellular cell signaling cascade, which leads to $Ca^{2+}$ mobilization[12, 21, 22]. These reports suggest, in part, an interaction between PAF and Rho kinase pathways in initiation of wide-ranging pulmonary vascular outcomes. Previous studies have shown that the Rho kinase inhibitor, Y-27632 decreases PAF-induced pressor responses in isolated perfused lungs[13]. The mechanism by which Rho kinase mediates PAF effects in vascular smooth muscles is not well understood and the SMC locale of ROCK effects has not been delineated. Whether responses originate from membrane-, cytosol-, or nuclear- bound receptors is not clearly defined. Thus it is necessary to examine the degree of PAFR binding in these three subcellular fractions of pulmonary vascular SMC. As a GPCR-linked receptor, PAF physiological and biochemical actions have been linked to some specific proteins in some cell types, where phosphorylation, internalization or hydrolysis have been implicated as responsible factors for quenching or diminishing PAFR-mediated response[23, 24, 25, 26, 27]. Thus it is necessary, as an initial step, to determine whether significant PAFR binding occurs in subcellular protein fractions other than the membrane. Rho Kinase is functionally linked to PAF–mediated pulmonary vascular reactivity. Our hypothesis is that PAFR-linked responses in fetal ovine pulmonary vascular smooth muscle cells (PVSMC): for instance; vasoconstriction, calcium release and proliferation, are in part mediated by Rho kinase signaling. The ovine species, due to its comparable size to human infants, is extensively used in the study of *in vivo* and *in vitro* physiology and pharmacology of perinatal pulmonary hemodynamics[28, 29, 30, 31, 32, 33, 34]. We carried out *in vitro* studies employing subcellular fractions of fetal ovine PVSMC to investigate the involvement of Rho kinase in PAFR binding and expression of some contractile proteins in the PAFR signaling pathway. We first conducted a saturation PAFR binding to the cells in normoxia and hypoxia, from which a working dose was selected to compare the effect of the Rho kinase inhibitor, Y-27632, the protein synthesis inhibitor, cycloheximide, and the PAFR inhibitor CV-3988 on PAF binding to its receptors in smooth muscle cells from pulmonary arteries (PA-SMC) and veins (PV-SMC) so as to gain an insight in the regulatory pathways occurring in pulmonary arteries and veins of the developing lung of fetal lamb. Rho kinase inhibitors Y-27632 and Fasudil (HA-1077) were also used to probe effect of PAF on expression of the contractile proteins myosin light-chain kinase (MLCK) and calponin. Effect of PAF and CV-3966 on CV-3988 PAF receptor gene expression by PA- and PV-SMC was also studied by qRT-PCR in normoxia and hypoxia.

## Materials and Methods

### Materials

The studies were approved by the Institutional Animal Care and Use Committee of the Lundquist Institute at Harbor-UCLA Medical Center. The authors do not have any financial conflict of interest with the vendors or companies associated with the reagents and supplies used in these studies. Pregnant ewes (146-148 d gestation, term being 150 d) were purchased from Nebekar Farms, Santa Monica, CA. Authentic standards of 1-O-hexadecyl-2-O-

acetyl-sn-glycero-3-phosphorylcholine [$C_{16}$-PAF (PAF)] and 1-O-hexadecyl-sn-glycero-3-phosphorylcholine (lyso-$C_{16}$-PAF) standards as well as (R)-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide (Y-27632) and 1-(5-isoquinolinesulfonyl) homopiperazine [Fasudil (HA-1077)] were purchased from Biomol (Plymouth Meeting, PA). Tritiated PAF $C_{16}$ ($^3$H-PAF-$C_{16}$) standard was purchased from Perkin Elmer (Wellesley, MA). (±)-[3-(N-Octadecylcarbamoyloxy)-2-methoxy]propyl 2-thiazolioethyl phosphate) (CV-3988) and the dicarboximide, 4-(2-hydroxyethyl)piperidine-2,6-dione (cycloheximide), Phenylmethysulfonyl fluoride (PMSF), leupeptin, pepstatin, bovine serum albumin (BSA), as well as antibody to α-smooth muscle actin were purchased from Sigma-Aldrich Company (St. Louis, MO). Antibody to PAF was purchased from Cayman Chemical (Ann Arbor, MI). Studies were done with freshly made reagents. Ecolite(+) liquid scintillation cocktail was purchased from MP Biochemicals (Irvine, CA). All other reagents and chemicals were purchased from Fisher Scientific (Santa Clara, CA) and as indicated when being described.

## Methods

### Preparation of pulmonary vascular smooth muscle cells (PVSMC).

Intrapulmonary vessels were isolated from freshly killed term fetal lambs. Smooth muscle cells were harvested from the freshly excised arteries and veins under sterile conditions as previously reported[21]. Cells were isolated from 2$^{nd}$ to 4$^{th}$ generation vessel segments of the arteries or veins and used at the 4$^{th}$ to 10$^{th}$ passage. Identity of the smooth muscle cells at each passage was characterized with a smooth muscle cell-specific monoclonal antibody SIGMA (St. Louis, MO). The SMC were devoid of endothelial cells and fibroblasts. Cell synthetic and proliferative phenotype did not change from 4$^{th}$ to 10$^{th}$ passages as has been shown in our previous reports[6, 21].

**Study conditions**: All cell culture studies were done in vitro on SMC from intrapulmonary arteries (PA-SMC) and veins (PV-SMC). Adherent cells were cultured in normoxia or hypoxia according to the specific experimental protocol.

***Normoxia conditions:*** Cells were studied in humidified incubator at 37 $^O$C aerated with 5% $CO_2$ 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% and $pO_2$ in culture media was maintained at 80-100 Torr.

***Hypoxia conditions:*** An incubator set at 37 $^O$C was first equilibrated for at least 3 h with a gas mixture of 2% $O_2$, 10% $CO_2$, and balance $N_2$ to maintain incubator culture media $pO_2$ <40 Torr, also monitored with TED 60T percent oxygen sensor, Teledyne Analytical Instruments (City of Industry, CA). The media blood gas values were determined on ABL700 blood-gas instrument, Radiometer America (Westlake, Ohio). After equilibration, cells were placed in this incubator for experimentation according to the specific experimental protocol. Cells were continuously aerated with the hypoxia gas mixture throughout the duration of the study.

### General Experimental protocols

***Culture of cells in normoxia or hypoxia:*** Arterial [PA-SMC, (arterial smooth muscle cells)] and venous [PV-SMC, (venous smooth muscle cells)] smooth muscle cells were seeded in culture dishes and grown to sub-confluence in 10% FBS culture medium containing a cocktail of antibiotics and antimycotic agents, in a humidified incubator aerated with 5% $CO_2$ in air at 37 $^O$C. For effect of normoxia on PAF binding to its receptor proteins, the sub-confluent cells at 80-90% confluence were cultured in normoxia conditions for 24 h more with the 10% FBS culture media. For effect of hypoxia the sub-confluent cells were placed in

an incubator pre-equilibrated for 3 h with a gas mixture of 2% $O_2$, 10% $CO_2$, balance $N_2$, to maintain $pO_2$ <40 Torr in the 10% FBS cell culture media. Cells were placed in this equilibrated incubator and cultured for 24 h more with continuous aeration with the hypoxia gas mixture.

**Protein isolation for PAF receptor (PAFR) binding assays**

a) **Saturation PAFR binding assay**: Sub-confluent cells were cultured in normoxia or hypoxia as described above under general experimental protocol. Membrane protein fractions were isolated from cells cultured in normoxia or hypoxia as we reported previously[21]. Briefly, the untreated cells were lysed with a hypotonic lysis buffer by bathing the cells in an ice-cold hypotonic lysis buffer and sonicating the lysed cells on ice for 1 min at 15 sec bouts and then the cell lysate was centrifuged at 9,000g for 10 min. The 9,000g supernatant was centrifuged at 4 $^{O}$C for 1 hr at 100,000g to pellet the membrane proteins. The 100,000g pellet was re-suspend in 50mM Tris buffer pH 7.2 containing 0.1mM PMSF and 5mM $MgCl_2$, 125 mM choline chloride, 0.25% BSA and 1.1M sucrose (the sucrose buffer) and spun again 4$^{O}$C for 1 hr at 100,000g to wash the membranes fraction and re-pellet membrane proteins which were then re-suspend in the sucrose buffer, stored in 0.2mL aliquots, kept at -80 $^{O}$C and used to measure the concentration effect of PAFR binding. Protein concentration was determined by the Bicinchoninic Acid (BCA) protein assay.

b) **Effect of PAF, rho kinase, cycloheximide and PAFR antagonist on PAF receptor protein synthesis:** Sub-confluent cells were cultured in normoxia or hypoxia as described above under general experimental protocol. The sub-confluent cells were treated separately with the following agents to investigate the inhibitory or stimulatory properties of the agent on PAF receptor protein synthesis. The agents were: a) 10nM of non-radiolabeled PAF standard; b) 10μM of the rho kinase inhibitor, Y-27632 or HA-1077; c) 30μM of cycloheximide, a protein synthesis inhibitor that has been used in our laboratories; and d) 1.0μM of CV 3988, a specific PAF receptor antagonist. After treatment, the treated arterial and venous smooth muscle cells were cultured for 24 h more in normoxia or hypoxia. The cell treatments were washed off with $Ca^{2+}$ and $Mg^{2+}$ free phosphate buffered saline and then subjected to cell lysis protocol with hypotonic lysis buffer[21]. Lysed cells were sonicated on ice for 1 min at 15 sec bouts then membrane, cytosolic [also identified as soluble fraction, (SF)], and nuclear protein fractions (NF) were isolated from cells cultured in normoxia or hypoxia. To isolate nuclear fraction, cell lysates were spun at 500g for 10 min in refrigerated centrifuge. The nuclear fraction pellet was re-suspended in the sucrose buffer for PAFR binding assay. Proteins in the soluble fraction were prepared by centrifuging the 500g supernatant at 9,000g for 10 min. The 9,000g supernatant was designated the soluble fraction and used to study PAFR binding. Then the 9,000g pellet was re-suspended in sucrose buffer and spun for 1 hr at 100,000g at 4$^{O}$C to pellet membrane proteins which were re-suspend in the sucrose buffer as with the nuclear fraction and used for PAFR binding assays. Protein concentrations were determined by the BCA protein assay. The prepared cell protein fractions were re-suspend in the sucrose buffer, stored in 0.2mL aliquots at -80 $^{O}$C and used to study PAFR binding.

**Specific protocols**

**PAF receptor (PAFR) binding assays for saturation binding and effect of treatment agents on proteins of cell protein fractions.** PAF receptor binding assay buffer was the 50 mM Tris buffer, pH 7.2, containing 0.1 mM PMSF, 5 mM magnesium chloride ($MgCl_2$), 125 mM choline chloride, 0.25% BSA, and 1.1mM sucrose (the sucrose buffer). Receptor binding assays were done at $4^OC$ for 24h with 100 µg protein/ml of assay buffer.

a) **Saturation PAFR binding assays:** For saturation binding, membrane proteins from normoxia or hypoxia cells was incubated for 24 h in normoxia or hypoxia according to cell protein origin, with different concentrations of non-radiolabeled $C_{16}$-PAF and 5 µCi per assay tube of $^3$H-PAF. After incubation for 24 hr at $4^OC$, $^3$H-PAF bound to its receptor was extracted on Whatman GF/C membrane filters using Whatman filter manifold and in-line vacuum system. The assay tubes were washed with calcium-free Tyrode's buffer, pH 6.4 containing in mM: NaCl, 137; KCl, 2.7; $NaHCO_3$, 11.9; $MgCl_2$, 1.0; $NaH_2PO_4$, 0.41; HEPES, 5.0; Glucose, 5.6; and BSA 0.25%, and transferred to the filtration manifold for extraction[3]

b) **Effect of PAF, Y-27632, cycloheximide and CV-3988 on PAFR binding to subcellular protein fractions:** For PAFR binding to cell protein fractions, membrane, soluble (SF) and nuclear fraction (NF) proteins from normoxia or hypoxia cells, 100µg protein/ml of assay buffer of proteins from a) the 10nM PAF treated cells; b) 10µM Y-27632 treated cells; c) 30µM of cycloheximide (CHX); and d) 1.0µM of CV 3988, a specific PAF receptor antagonist, was incubated for 24 hr at $4^OC$ in normoxia or hypoxia according to cell protein origin, together with 5 µCi per assay tube of $^3$H-PAF. After incubation for 24 hr at $4^OC$, $^3$H-PAF bound to its receptors was extracted on Whatman GF/C membrane filters using Whatman filter manifold and in-line vacuum system. The assay tubes were washed with calcium-free Tyrode's buffer, pH 6.4 containing in mM: NaCl, 137; KCl, 2.7; $NaHCO_3$, 11.9; $MgCl_2$, 1.0; $NaH_2PO_4$, 0.41; HEPES, 5.0; Glucose, 5.6; and BSA 0.25%, and transferred to the filtration manifold for extraction[3].

For both protocols a) and b), cell-bound PAF radioactivity on the membrane filters, was detected by scintillation spectrometry (Beckman Coulter Instruments, Fullerton, CA). Receptor bound PAF was quantified as PAF bound in fmol/µg protein**.** For all lysed cells, membrane, cytosol, and nuclear proteins were prepared and PAF receptor binding assay were done on all the fractions to correlate PAFR protein synthesized with observed locales. Proteins were also prepared to measure expression of proteins of interest by Western blotting.

**PAF receptor binding to adherent cells**

Cells were cultured to sub-confluence and then used for the binding assays. Briefly, arterial or venous cells were washed with PBS followed with the PAF receptor assay buffer. To study effect of Y-27632 on PAFR binding to PA-SMC or PV-SMC, washed cells were pre-incubated for 30 min at $37^OC$ in normoxia or hypoxia, with 10µM of Y-27632 or 10nM of non-radiolabed PAF standard and Then 5µCi of [$^3$H]-acetyl-$C_{16}$-PAF ($^3$H-PAF) was added to the cells and incubated for 30 min more at $37^OC$ in normoxia or according to the specific protocol. To study effect of CV-3988 on PAFR binding to arterial or venous smoothe muscle cells, washed cells were pre-incubated for 30 min at $37^OC$ in normoxia or hypoxia, with 1.0µM of CV-3988 or 10nM of non-radiolabed PAF standard and then 5µCi of [$^3$H]-acetyl-$C_{16}$-PAF ($^3$H-PAF) was added to the cells and incubated for 30 min more at $37^OC$ in normoxia or according to the specific protocol. After 30 min

incubation in normoxia or hypoxia, unbound $^{3}$H-PAF was washed off with ice-cold PBS followed by washes with 5% trichlroroacetic acid. Then a mixture of 154 mM saline and 5 mM EDTA was added to the cells and incubated on ice for 45 min. $^{3}$H-PAF bound to its receptor was extracted on Whatman GF/C membrane filters using Whatman filter manifold and in-line vacuum system. The culture flask or dish was washed with calcium-free Tyrode's buffer, pH 6.4 containing in mM: NaCl, 137; KCl, 2.7; $NaHCO_3$, 11.9; $MgCl_2$, 1.0; $NaH_2PO_4$, 0.41; HEPES, 5.0; Glucose, 5.6; and BSA 0.25%, and transferred to the filtration manifold for extraction. Cell-bound PAF radioactivity was quantified by scintillation spectrometry (Beckman Coulter Instruments, Fullerton, CA)[3, 21, 22].

**Western blotting**

***Preparation of proteins for Western analysis:*** Western blotting was done according to previous reports[6, 8, 21]. Briefly, after incubation in hypoxia or normoxia, cells were washed with PBS and lysed with a modified 40 mM HEPES hypotonic lysis buffer, pH 7.4, containing the following; 1 mM EGTA, 4 mM EDTA, 2 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM PMSF, 5 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µM 4-(2-aminoethyl) benzene sulfonyl fluoride, 200 mM sodium fluoride, 20 mM sodium pyrophosphate, 0.2 mM sodium vanadate and, 0.1 mg/ml trypsin inhibitor. Proteins were recovered from lysed cells by centrifugation at 1500g for 15 min in refrigerated Eppendorf centrifuge and stored in 0.2 ml aliquots at -80$^{o}$C and used for Western blotting.

***Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE):*** Proteins were subjected to Coomassie blue quantitation before use in Western blotting with some modification of published methods[8, 21, 22, 23]. SDS-PAGE was run on 4-12% glycine gels (Lonza). The signals were developed using Amersham ECL Western Blot detection kit and then exposed to X-ray film. Bands corresponding to MLCK, Calponin and PAFR protein or other proteins of interest were scanned with an UnscanIT program to quantify blot density.

**Study of PAF receptor gene expression by real time polymerase chain reaction**

Arterial and venous smooth muscle cells of fetal lambs were cultured in normal incubator conditions (5% carbon dioxide in air) and in hypoxia and stimulated with 10nM PAF or with 1.0µM of the PAFR inhibitor, to study PAF receptor gene expression by quantitative real time PCR (qRT-PCR). 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 Step One Plus real time PCR System (Applied Biosystems, Foster City, CA). Briefly, reverse transcription was accomplished with Applied Biosystems High Capacity RNA-cDNA Master Mix (cat # p/n 4390715) program as follows: step 1, 25$^{O}$C for 5 min; step 2, 42$^{O}$C for 30 min; step 3, 85$^{O}$C for 5 min; and step 4, hold at 4$^{O}$C[8]. 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$^{O}$C 1 cycle; step 2, 95$^{O}$C 10 min 1 cycle: 95$^{O}$C 15 sec, 59$^{O}$C 1 min, 40 cycles (annealing step/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', bp 98-117, 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 primer 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 qRT-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 which was used as the reference, and qPCR data are presented as $\Delta C_t$, means ± 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. In this report, we used $\Delta t$ instead of $\Delta\Delta t$ so as to show in an appreciable method, the PAFR gene expression under control conditions compared to expression under stimulated conditions[8].

**Data analysis:** All numerical data are means ± SEM. In all instances where radioisotope was used, background radioactivity was subtracted before quantifying radioactivity. For the saturation binding, the nonspecific binding was subtracted for all data and then the specific binding data was plotted to show the saturation binding isotherms for each condition. To establish an "n" value in statistics, cells from each animal (fetal lamb) and for each condition were studied at least in triplicates, the results were averaged to indicate an "n" =1. Therefore, an "n=4 or 6" statistics means that 4-6 different fatal lambs were studied as describe above. Then data were subjected to statistical analysis. To compare data from normoxia versus hypoxia, a two-tailed t-test was used. For multiple comparisons, an ANOVA was used followed by Tukey posthoc test (GraphPad Prism, San Diego, CA). Results were considered significant at $p < 0.05$.

## Results

### Effect of hypoxia on saturation PAFR binding to PA-SMC and PV-SMC

We first compared effect of different PAF concentrations on PAF receptor binding to membrane proteins from arterial and venous cells in normoxia figure 1a, and hypoxia, figure 1b. In both smooth muscle cell types, PAF receptor binding was concentration-dependent. The plots represent specific PAFR binding and the Scatchard plots, shown as insets to the respective saturation binding curves, for the specific PAFR binding in normoxia and hypoxia. Hypoxia increased PAF binding to its receptors in membrane proteins from both arterial and venous cells in a concentration-dependent manner. Comparing binding to membrane proteins of each cell type during normoxia and hypoxia, PAFR binding to proteins in normoxia was significantly lower than binding in hypoxia. Comparing binding to membrane proteins from arterial cells to membrane proteins from venous cells, binding to venous membrane proteins was significantly greater than for proteins from arterial cells both in normoxia and hypoxia.

**Figure 1.** Saturation binding isotherms of PAFR binding to membrane fractions PA-SMC and PV-SMC

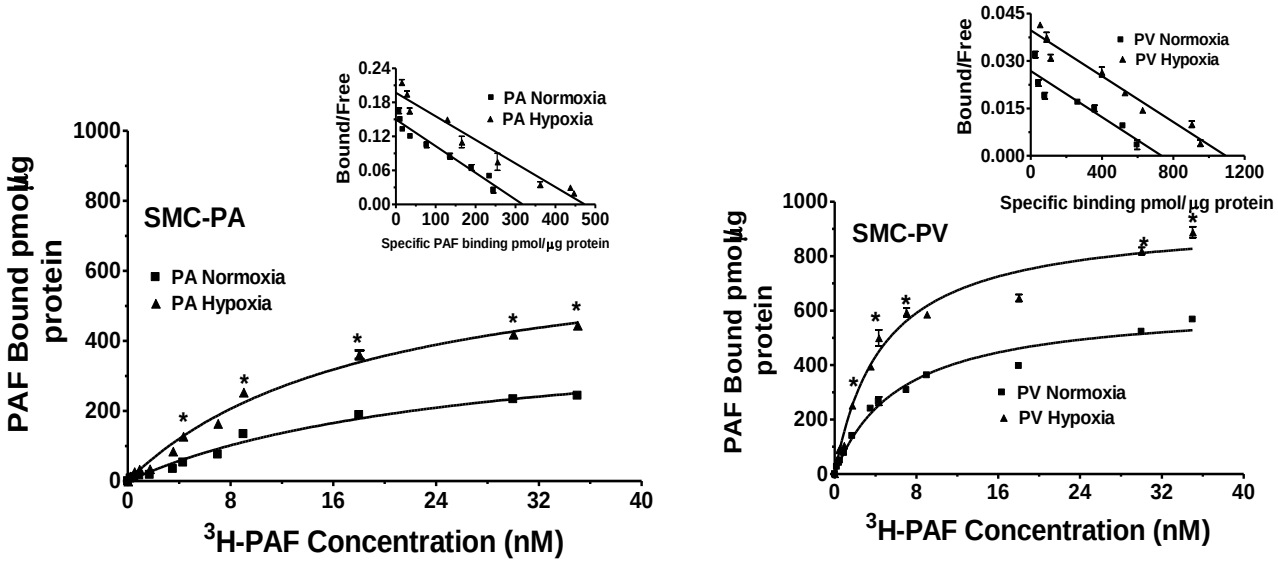



**Figure 1:** PAF receptor binding isotherms on membrane proteins of PA-SMC and PV-SMC in normoxia (closed squares) and hypoxia (closed triangles). Sub-confluent cells were cultured in normoxia or hypoxia without stimulation. Membrane proteins were prepared and used to assay saturation PAFR binding. Data are means ± SEM, n = 6 and analyzed as described under data analysis. In both PA-SMC, figure 1a, and PV-SMC figure 1b, binding in hypoxia was greater than binding in normoxia. The scatchard plot of each cell binding isotherm is shown with each binding isotherm. The statistics are *p <0.05, different from normoxia.

The PAF receptor density ($B_{max}$) and the binding dissociation constants ($K_D$) in arterial and venous cell membrane proteins are shown in figure 2. The $B_{max}$ (fmol/µg protein) in normoxia and hypoxia is shown in figure 2a. In arterial cell proteins from normoxia cells, $B_{max}$ was 487.2±37.2. In proteins from hypoxia cells, the value was 700.6±31.6. The receptor $B_{max}$ for proteins from hypoxia cells was 30% greater than proteins from normoxia cells. For venous cell proteins, PAF receptor $B_{max}$ was 624.4±17.2 in normoxia and 939.1±25.0 in hypoxia. The receptor $B_{max}$ for proteins from hypoxia cells was ~35% greater than for normoxia proteins. In general, PAF receptor $B_{max}$ in venous cell proteins was higher than for arterial cell proteins in normoxia and hypoxia conditions.

**Figure 2:** PAFR $B_{MAX}$ and $K_D$ measured in PA-SMC and PV-SMC

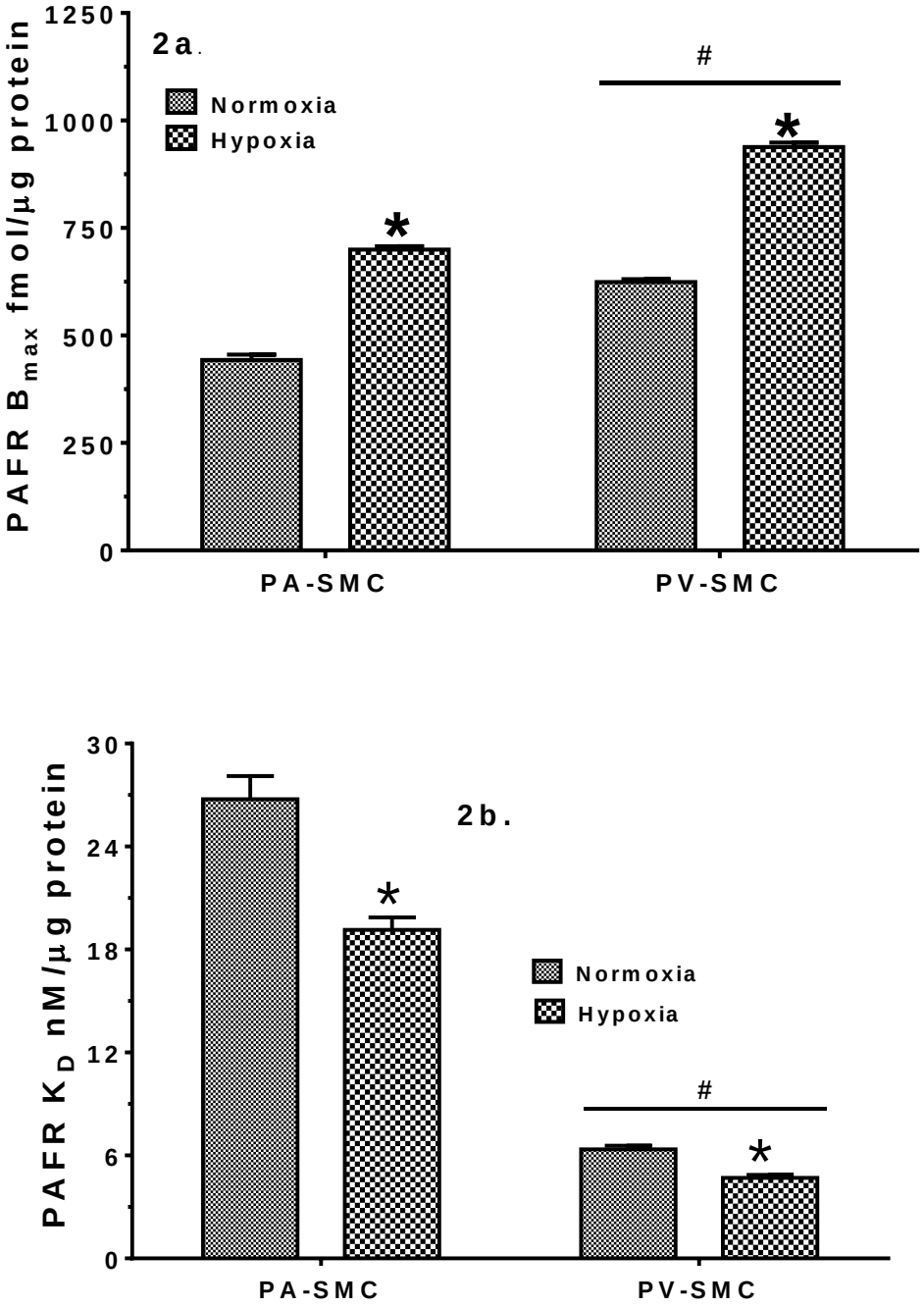



**Figure 2:** PAF receptor density ($B_{max}$), and dissociation constant PAF binding to PA-SMC and PV-SMC in normoxia and hypoxia. Receptor binding isotherms were subjected to binding Scatchard statistical analysis for $B_{max}$ and $K_D$ quantification. Data are means ± SEM, n = 6. PAF receptor density, $B_{max}$, figure 2a, was higher in hypoxia for both cell types and whether in normoxia or hypoxia, PAF receptor density was higher in PV-SMC. PAF receptor dissociation constant, $K_D$, figure 2b was lower in hypoxia for both cell types. The $K_D$ for PAFR binding was lower in PV-SMC than for PA-SMC in both normoxia and hypoxia. The statistics are: *p <0.05, different from normoxia; #p <0.05, different from PA-SMC.

The dissociation constant ($K_D$) of PAF receptor binding (nM/μg protein) to receptors in arterial and venous cells are shown in figure 2b. In proteins from arterial cells from normoxia, the $K_D$ was 30.4±4.7. In proteins from hypoxia cells, the value was 19.2±1.8. The $K_D$ in proteins from hypoxia cells was 40% lower than $K_D$ from normoxia proteins. In proteins from venous cells in normoxia, $K_D$ was 6.4±0.5. In hypoxia cells, the value was 4.7±0.4. The receptor binding $K_D$ in proteins from hypoxia cells was 25% lower than for proteins from normoxia. In general, PAF receptor binding $K_D$ in venous cell proteins was lower than for arterial cell proteins in normoxia and hypoxia, 80% and 75% lower in normoxia and hypoxia, respectively.

**Effect of rho kinase inhibitor, Y-27632, cycloheximide and CV-3988 on PAF receptor binding to proteins from PA-SMC and PV-SMC cultured in normoxia or hypoxia**

Membrane, cytosolic [soluble fraction (SF)], and nuclear fraction (NF) proteins were prepared from cells co-incubated with the agents listed above for 24 h and used to measure PAF binding. Figure 3 shows the effect of cycloheximide, the Rho kinase inhibitor, Y-27632, and the PAFR antagonist, CV-3988, on PAF receptor binding compared to control conditions. Concentrations used are as described in general methods above. Cells were cultured with these agents for 24 h in normoxia or hypoxia. Specific PAF receptor binding (fmol/μg protein, means ± SEM) was determined in arterial and venous cells in nromoxia and hypoxia. PAF binding to its receptors in membrane proteins from arterial cells in normoxia, figure 3a, was 492±60.0 which decreased to 94±15 and 75±8 in soluble (SF) and nuclear (NF) fractions respectively. PAF binding to its receptors in soluble and nuclear fraction proteins was significantly less than binding to membrane proteins. PAF binding to receptors in membrane proteins from cyloheximide, Y-27632, and CV-3988 treated cells were 285±40, 117±8, and 116±14 respectively. Binding to soluble and nuclear fractions of cycloheximide cells were 159±45 and 44±6 respectively; binding to soluble and nuclear fractions of Y-27632 were 47±6 and 20±4 respectively; for CV-3988 treated cells binding were 33±6 and 35±9 respectively. PAF binding to soluble and nuclear fraction for cells treated with these agents were significantly less than binding to membrane fractions of control and treated cells, and then each of the three treatments: cycloheximide, Y-27632, and CV-3988 decreased PAF binding to proteins in the respective fractions. Although the intent of this study was not to discover which treatment is a more potent inhibitor, in general, Y-27632 and CV-3988 produced similar inhibitory effect on PAF receptor binding.

In proteins from arterial cells in in hypoxia, PAF binding to receptors in membrane proteins, figure 3b, was 680±60 which decreased to 196±10 in SF, and 140±27 in NF. PAF binding to its receptors in SF and NF proteins was significantly less than binding to membrane proteins. PAF binding to receptors in membrane proteins from cycloheximide, Y-27632, and CV-3988 treated cells were 349±40, 106±25, and 169±30 respectively. Binding to SF and NF of CHX cell were 159±5 and 47±6 respectively; binding to SF and NF fractions of Y-27632 were 49±15 and 18±5 respectively; for CV-3988 treated cells binding were 73±16 and 39±12 respectively. PAF binding to SF and NF fractions were significantly less than binding to membrane fractions and then each of the three treatments: cycloheximide, Y-27632, and CV-3988 decreased PAF binding to proteins in the respective fractions compared to binding to proteins from control cells. A casual observation of effect of these treatments on PAFR binding to proteins from arterial cells from hypoxia, shows that Y-27632 treatment produced greater inhibitory effect of PAFR

binding than cycloheximide or CV-3988 treatments.

**Figure 3a and 3b:** PAF receptor binding to membrane, cytosol (SF) and nuclear protein fractions of PA-SMC
**Figure 3c and 3d:** PAF receptor binding to membrane, cytosol (SF) and nuclear (NF) protein fractions of PV-SMC

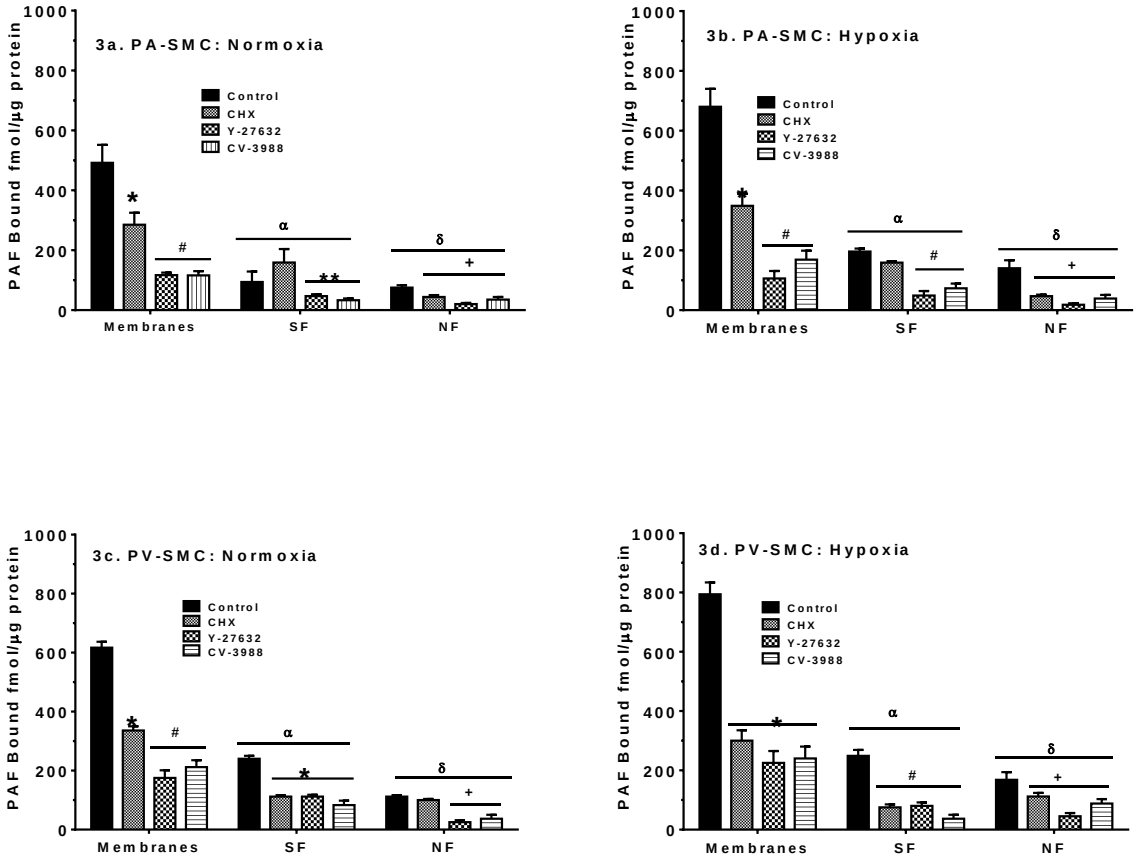



**Figure 3:** Effect of PAF, cycloheximide (CHX), Rho kinase inhibitor (Y-27632), and CV-3988 treatment on PAFR binding to membrane, soluble (SF) and nuclear (NF) fractions of PA-SMC and PV-SMC of the fetal lamb lungs cultured in normoxa and hypoxia. Sub-confluent cells were incubated for 24 hr with buffer alone (Control), or separately with 10.0µM Y27632, 30.0µM CHX, and 1.0µM CV-3988, in normoxia or hypoxia. Proteins were prepared and used to assay PAFR binding. Membranes, SF, and NF were purified by differential centrifugation and assayed for PAF receptor binding. Data are means ± SEM, n = 6 and analyzed as described under data analysis. In PA-SMC during normoxia, figure 3a, PAFR binding (fmol/µg protein) to membrane proteins in control was 492±60.0 which decreased significantly with CHX, Y-27632, and CV-3988 treatments. In PA-SMC during hypoxia, figure 3b, binding to membranes fractions of control was 680±60, which also decreased significantly with CHX, Y-27632, and CV-3988 treatments. For both normoxia and hypoxia, PAFR binding to SF for each of the treatments was significantly less than binding to membranes, and then binding to NF fractions was significantly less than binding to membrane and soluble fractions from each of the treatments. In PV-SMC during normoxia, figure 3c, binding to membranes fractions of control was 617±20, which decreased significantly with CHX, Y-27632, and CV-3988 treatments. In PV-SMC during hypoxia, figure 3d,

binding to membranes fractions of control was 794±50, which also decreased significantly with CHX, Y-27632, and CV-3988 treatments. As in PA-SMC, for both normoxia and hypoxia, PAFR binding to SF for each of the treatments was significantly less than binding to membranes, and binding to NF fractions was significantly less than binding to membrane and soluble fractions from each of the treatments. In both cell types and in all study conditions, CHX, Y-27632, and CV-3988 inhibited PAFR binding and then binding to the subcellular fractions were significantly less than binding to membranes. Statistics are: *p <0.05, different from binding to Membranes in Control; $^{\#}$p <0.05, different from binding to Controls and CHX; **p <0.05, different from binding to Control and CHX in normoxia PA-SMC; +p <0.05, different from Control in NF control; αp <0.05, different from membranes for all treatments; $^{\delta}$p <0.05, different from membranes and SF for all treatments.

In venous cells in normoxia, PAFR binding to membrane proteins, figure 3c, was 617±20 which decreased to 240±10 in SF, and 115±5 in NF. As in arterial cell proteins above (3a and 3b), PAF binding to its receptors in SF and NF proteins was significantly less than binding to membrane proteins. PAF binding to receptors in membrane proteins from cycloheximide, Y-27632, and CV-3988 treated cells were 336±14, 175±26, and 212±23 respectively. Binding to SF and NF fractions of cycloheximide cells were 112±5 and 100±4 respectively; binding to soluble and nuclear fractions of Y-27632 cells were 112±6 and 25±7 respectively; for CV-3988 treated cells values were 83±15 and 37±13 in SF and NF proteins respectively. PAF binding to SF and NF proteins were significantly less than binding to membrane fractions and then each of the three treatments: cycloheximide, Y-27632, and CV-3988 decreased PAF binding to proteins in the respective fractions. A casual observation also reveals that the three treatments produced unique inhibitory profile of PAFR binding to proteins from of each of the subcellular fractions.

In proteins from venous cells in hypoxia, PAFR binding to receptors in membrane proteins, figure 3d, was 794±40 which decreased to 249±20 in SF, and 168±26 in NF. PAF binding to its receptors in SF and NF proteins was significantly less than binding to membrane proteins. PAF binding to receptors in membrane proteins from cycloheximide,, Y-27632, and CV-3988 treated cells were 300±40, 225±40, and 240±45 respectively. Binding to SF and NF fractions of cycloheximide cells were 75±10 and 112±12 respectively; binding to SF and NF for Y-27632 proteins were 80±12 and 45±11 respectively; for CV-3988 treated cells binding were 37±13 and 88±15 respectively. PAF binding to soluble and nuclear fraction were significantly less than binding to membrane fractions and then each of the three treatments: cycloheximide, Y-27632, and CV-3988 decreased PAF binding to proteins in the respective fractions. Their inhibitory profiles of binding to SF and NF proteins compared to proteins from controls is unique to each inhibitor and the subcellular fraction.

Generally, PAFR binding in membrane proteins from hypoxia cells is greater than binding to proteins from normoxia cells, but this observation is not consistently exact for binding to the other subcellular fractions. PAFR binding to membrane protein from venous cells in normoxia and hypoxia are greater than binding to proteins from arterial cells in normoxia and hypoxia.

**PAF receptor binding to adherent cells**

PAFR binding (fmol/$10^6$ cells, means ± SEM) to adherent arterial and venous cells are shown in figure 4. In arterial cells, figure 4a, 10μM Y-27632 did not produce any significant inhibition of PAF binding to its receptors in normoxia or hypoxia. In venous cells, in normoxia and hypoxia, figure 4b; 10μM Y-27632 produced no significant change in PAFR binding, some increase in binding was detected, but this increase did not attain statistical significance

(p=0.053). In hypoxia, some inhibition in binding was detected, but the inhibition did not attain statistical significance, 36.6±4.7 and 31.0±6.1 for control and Y-27632 treated cells respectively (p=0.092). However, treatment of arterial cells with 1.0µM CV-3988, figure 4c, resulted in significant inhibition of PAFR binding, 50% and 45% in normoxia and hypoxia respectively. Also in venous cells, figure 4d, 1.0µM of CV-3988 produced 54% inhibition of PAFR binding in normoxia (20.6±3.7 vs 9.9±2.6) and 62% inhibition in hypoxia (36.6±3.7 vs 13.9±4.3 in hypoxia). Thus, in both arterial venous cells, CV 3988 inhibited PAFR receptor binding, while Y-27632 did not inhibit binding to adherent cells within 30 min in culture conditions. Therefore Y-27632 cannot satisfactorily inhibit PAF binding to pre-synthesized PAF receptor proteins in pulmonary vascular smooth muscle cells.

**Figure 4a, 4b, 4c, and 4d**: PAFR binding to adherent PA-SMC and PV-SMC studied at 30 min incubation

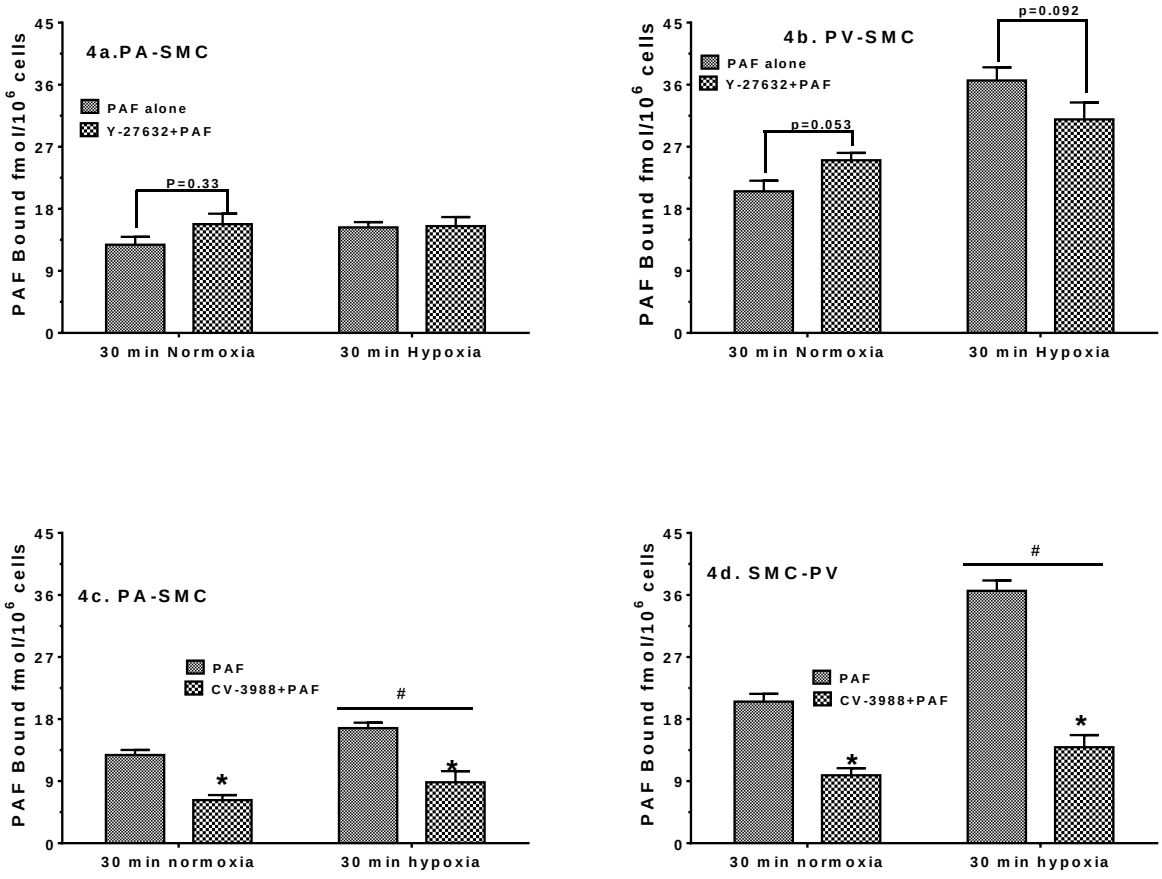



**Figure 4:** Effect of PAF and Y-27632 on PAFR binding to adherent PA-SMC and PV-SMC. Sub-confluent cells were treated with 10nM PAF alone or with 10µM Y-27632 or CV-3988 and pre-incubated for 30 min in normoxia or hypoxia. Then 5µCi of $^{3}H$-$C_{16}$PAF was added to all samples and incubated for 30 min more in normoxia or hypoxia. Data are means ± SEM, n=6. In PA-SMC, figure 4a, 30 min incubation with Y-27632 produced no significant change in PAFR binding to the cells within the 30 min compared to PAF treatment alone. In PV-SMC, in normoxia and hypoxia, figure 4b; 10µM Y-27632 produced no significant change in

PAFR binding. In PA-SMC 4c and 4d, sub-confluent cells were pulsed for 30 min with 10nM PAF or 1μM CV-3988 and incubated for 30 min in normoxia or hypoxia. Then 5μCi of $^{3}H$-$C_{16}$PAF was added incubated to all samples and incubated for 30 min more in normoxia or hypoxia as in with Y-27632 treated cells. In both PA-SMC, figure 4c and PV-SMC, figure 4d, CV-3988 produced significant inhibition of PAFR binding in normoxia and hypoxia. The statistics are: *p <0.05, different from PAF alone; #p <0.05, different from 30 min normoxia.

**Rho kinase modulation of expression of specific contractile proteins by arterial and venous smooth muscle cells.**

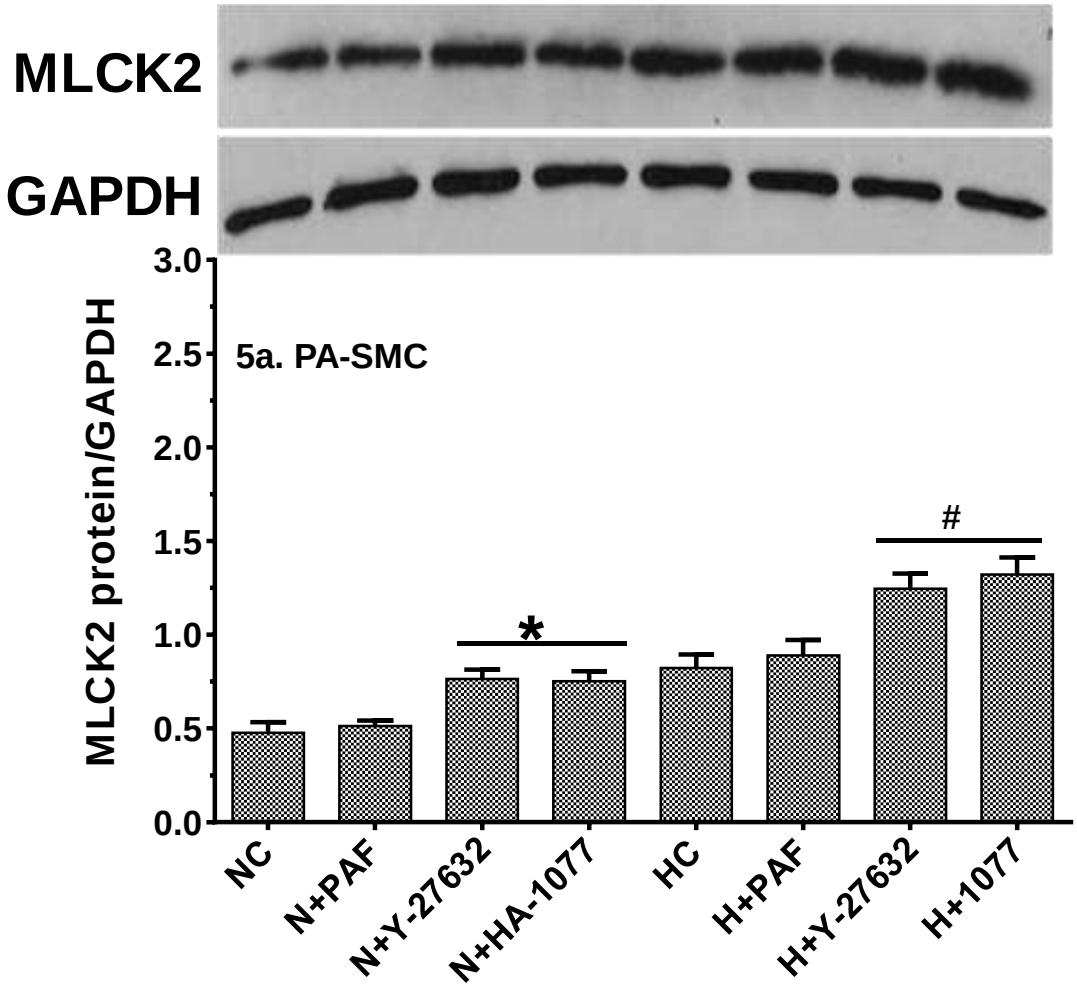
Proteins were isolated from arterial and venous cells cultured in normoxia (N) or hypoxia (H) for 24 h with the rho kinase inhibitors, 10μM each of Y-27632 and HA-1077. Expression of myosin light chain kinase (MLCK2), and Calponin proteins were probed by Western blotting and all quantified to expression of GAPDH internal standard. HA-1077 is another rho kinase inhibitor which has been shown to modulate PAF receptor expression in arterial and venous smooth muscle cells of perinatal lambs (Renteria et al, 2013). We wished to investigate involvement of PAF, hypoxia and rho kinase in modulating expression of these contractile proteins, MLCK2 and Calponin, by ovine fetal pulmonary smooth muscle cells. Figure 5a shows effect of PAF, hypoxia, Y-27632 and HA-1077 on expression of MLCK2 by arterial cells. PAF treatment of cells in normoxia did not alter MLCK2 expression compared to control conditions in normoxia. On the other hand, bothY-27632 and HA-1077 increased MLCK2 expression by 40%, with no difference in augmentation of expression by the rho kinase inhibitors, Y27632 and HA1077. Hypoxia increased MLCK2 expression compared to expression in control conditions in normoxia and also by PAF in control conditions in normoxia, Treatment with Y-27632 or HA-1077 also augmented MLCK2 expression compared to effect of hypoxia on protein expression, but with no difference between effects of the two inhibitors as in normoxia conditions. In venous cells figure 5b, PAF treatment of cells during normoxia led to over 3-fold decrease in MLCK2 expression. Treatment with the rho kinase inhibitors increased MLCK2 expression compared to effect of PAF. However, MLCK2 expression was still 40% less than expression by normoxia control conditions, with no difference in effect of Y-27632 or HA1077. Compared to normoxia control conditions, Hypoxia caused significant decrease in MLCK2 expression compared to normoxia conditions. PAF treatment increased MLCK2 expression compared to hypoxia conditions alone, but expression was still less than for control conditions in normoxia. Unlike in arterial cells, HA-1077 increased MLCK2 expression by 2-fold compared to effect of hypoxia alone or Y-27632. Of interest is the fact that MLCK expression in control conditions was significantly greater than the effect of the treatments in normoxia or hypoxia

With Calponin expression in arterial cells, figure 5c, PAF treatment increased Calponin protein expression in normoxia over control conditions. Treatment with Y-27632 or HA-1077 further increased Calponin expression by 40% over PAF effect alone, but there was no difference between effects of Y-27632 and HA-1077 in expression of Calponin. In hypoxia, PAF treatment also increased Calponin protein expression over control conditions in hypoxia. Also treatment with Y-27632 or HA-1077 increased Calponin expression over PAF effect, but there was no difference between effect of Y-27632 and HA10-77. In general, Calponin expression in hypoxia was greater than expression in normoxia both under control conditions and on treatment with the study agents. In venous smooth muscle cells, figure 5d, PAF treatment in normoxia caused a 25% decrease in Calponin expression compared to

control conditions. However, while Y-27632 increased Calponin expression, effect of HA-1077 on Calponin expression was not as dramatic as the effect of Y-27632., but unlike in arterial cells, these rho kinase inhibitors produced different stimulatory effect in Calponin expression in venous cells, effects which are about similar to the observations on MLCK2 expression, with Y27632 effect being significantly greater than effect of HA-1077. In hypoxia, PAF treatment increased Calponin expression over the control conditions in hypoxia. Treatment with Y-27632 and HA-1077 each decreased Calponin expression compared to effect of PAF treatment, with Y27632 producing greater inhibition of Calponin expression than HA-1077. As was observed in MLCK2 expression in arterial cells, Calponin expression did not present a consistency in profile of expression in venous cells.

**Figure 5a, and 5b:** Effect of PAF and Rho kinase inhibitors on MLCK2 protein expression by PA-SMC and PV-SMC



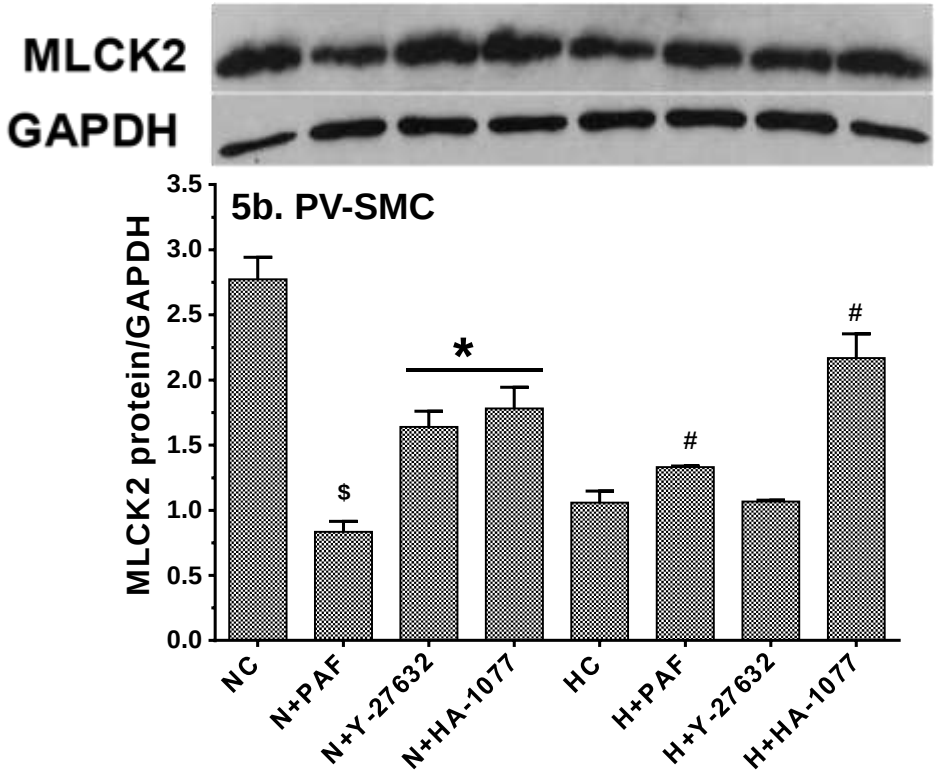



**Figure 5:** Effect of Rho kinase inhibitors on stimulation of MLCK2 and Calponin protein expression. Serum starved cells were pre-incubated for 2 hr with 10% FBS alone or 10% FBS plus 10µM each of Y-27632 and HA 1077 and then 10nM PAF was added to all cells and incubation continued for 24 hr more in normoxia or hypoxia. Proteins were extracted and probed for MLCK2 and Calponin protein expression by Western blotting and quantified against GAPDH internal standard. Data are means ± SEM, n = 4. The control in normoxia (NC, Control) or hypoxia (HC, Control) were incubated in 10% FBS alone. Compared to control conditions, in PA-SMC, figure 5a, PAF increased MLCK2 expression in hypoxia, but no change in normoxia. Y27632 and HA-1077 increased MLCK2 expression in normoxia and hypoxia. In PV-SMC, figure 5b, profile of MLCK2 expression was not clearly defined; with very high expression in normoxia control (NC). Y-27632 and HA-1077 tended to increase expression compared to effect of PAF alone. For Calponin expression compared to control conditions. In PA-SMC, figure 5c, expression increased with stimulation in both normoxia and hypoxia. In PV-SMC, however, profile of expression was not clearly defined as was observed in MLCK2 expression (figure 5b), expression increased with Y-27632 in normoxia, but not in hypoxia. PAF increased expression in hypoxia, but not in normoxia. The statistics are: *p <0.05, different from controls in normoxia (NC) or hypoxia (HC); #p <0.05, different from PAF treatment.

**PAF receptor mRNA expression by arterial and venous smooth muscle cells**

Effect of hypoxia, 10nM PAF and 1µM PAFR antagonist CV 3988 on PAFR gene expression is shown in Figure 6; Panel A, normoxia and Panel B, hypoxia. In normoxia, PAFR gene expression was higher in venous cells than arterial cells. There was no difference in PAFR gene expression by arterial and venous cells following treatment with 10nM PAF, but the exogenous PAF treatment increased PAFR gene expression in arterial cells. Interestingly, treatment of cells with 1µM of CV-3988, increased gene expression by both cell types, but more by venous cells. In hypoxia, there was no difference in gene expression by both cell types in the three treatment conditions studied. However, expression by arterial cells was greater than by venous cells.

Figure 6: PAF receptor gene expression by stimulated PA-SMC and PV-SMC

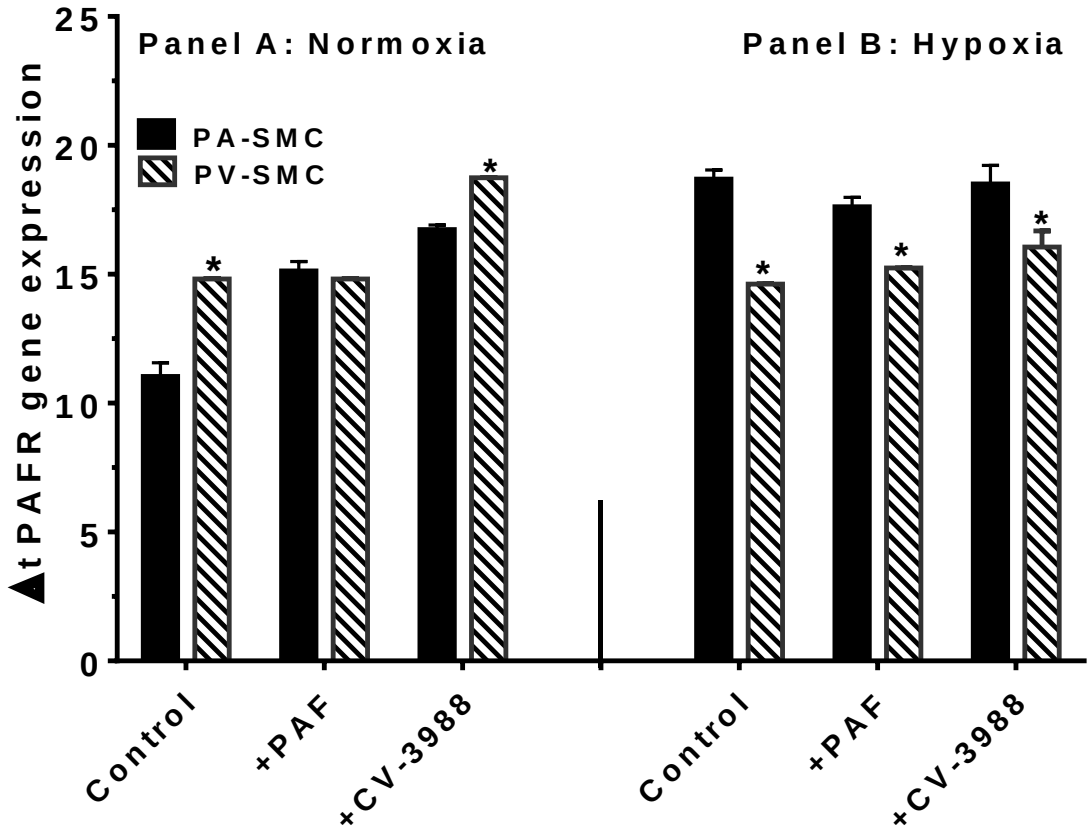



**Figure 6:** Stimulation of PAFR gene expression in PA-SMC and PV-SMC in normoxia and hypoxia studied by qRT-PCR. RNA was prepared from each of the cell types cultured in normoxia or hypoxia and subjected to quantitative RT-PCR (qRT-PCR). Gene expression was normalized to expression of GAPDH internal standard. Data are means ± SEM. In normoxia, PAFR gene expression was higher in PV-SMC in control conditions. PAF stimulated gene expression equally in PA-SMC and PV-SMC. Treatment with CV-3988 increased gene expression in both PA-SMC and PV-SMC, but greater expression PV-SMC. In general, hypoxia increased PAFR gene expression under all conditions compared to control condition in normoxia and then expression by PA-SMC was greater than by PV-SMC. *p <0.05, different from PA-SMC

**Discussion**

During physiological growth and development, proliferation of pulmonary vascular smooth muscle cells (PVSMC) plays an important role in normal growth of the vascular system [33]. Platelet activating factor (PAF), an endogenous lipid mediator, activates cell growth by autocrine and paracrine mechanisms [36]. We have examined the possible role of PAF as an upstream stimulus for vasoconstriction which is an important pulmonary vascular response of the fetal ovine species. We found that PAF exhibited a saturation binding profile in proteins from arteries and veins of fetal lambs and the saturation binding profile was significantly upregulated by hypoxic conditions, a physiological state of the fetal pulmonary system *in utero*. In this study, PAF binding was measured in, soluble fraction (SF) and nuclear

fraction (NF) of the smooth muscle cells. Binding to SF and NF, in normoxia and hypoxia proteins were significantly less compared to binding to membrane fractions under the same experimental conditions. The lamb is used extensively to study pulmonary physiology and pharmacology during the perinatal period[3, 6, 29, 29, 31, 34, 37, 39]. PAF is a potent modulator of the pulmonary circulation of sheep, which is a widely used model to study the vascular effects of PAF[3, 6,37, 39,] therefore our findings of profile of PAFR binding to its receptors in SF and NF proteins offer a new information for consideration to physiologists and pharmacologist undertaking studies of perinatal pulmonary responses.. The rho kinase inhibitor, Y-27632, attenuated PAFR binding to proteins from cells pre-incubated for 24 hr with the inhibitor, but did not inhibit binding to adherent cells incubated for 30 min in normoxia or hypoxia. This is a novel pieces of information explaining the mechanism of rho kinase modulation of PAFR-mediated responses in lung SMC, and suggests that pharmacologically, rho kinase inhibitor will be impacting on nascent protein synthesis in situ rather than on pre-synthesized proteins

**Saturation PAFR binding to membrane fractions of arterial and venous smooth muscle cells**

In previous studies, we have employed PAFR binding isotherms to characterize PAF binding to its receptors in pulmonary venous smooth muscle cells (PV-SMC) of fetal lambs[21, 39], and binding to perinatal lung membranes[23]. Data from these studies have greatly aided in our understanding of the physiological role of PAF in perinatal pulmonary adaptation. A commensurate characterization of PAFR binding to pulmonary arterial SMC has not been undertaken. We used direct radioligand binding to describe specific binding of PAF to its receptors in pulmonary arterial SMC. The results show that PAF binds specifically to its receptors in arterial SMC with a similar profile as in venous SMC, although the binding isotherms of the two SMC are quantitatively different. As shown in Figure 1, hypoxia upregulated PAFR binding to membrane proteins from both SMC, with binding to venous SMC being significantly higher in both normoxia and hypoxia. Binding of PAF to SMC membrane of each age type showed comparative high affinity, with a $K_D$ (nM/µg protein) in normoxia of 6.4 in venous SMC compared to 30.4 in arterial SMC; in hypoxia, and $K_D$ of 4.7 in PV-SMC and 19.2 in PA-SMC was measured. The smaller $K_D$ values in PV-SMC in normoxia and hypoxia indicate a greater binding affinity of PAF to its receptor in the venous SMC, a condition which is desirable for PAF to maintain congenial pulmonary vascular resistance in utero. Thus the larger $K_D$ value in normoxia and hypoxia point to a more weakly PAF interaction with its receptors in arterial SMC. In a similar study with fetal ovine lung membranes, and lung membranes of newborn lamb <90 min old, the $K_D$ of binding to fetal lung membranes was significantly less than that for newborn lamb <90 min old[23], which is in support of the need for a high PAFR binding affinity in fetal lungs to maintain the obligatory high pulmonary vascular resistance. In the present study, we also found that PAFR binding to the two cell types in normoxia and hypoxia was saturable with different receptor densities ($B_{max}$). In venous SMC, $B_{max}$ (fmol/µg protein) was 624.4 and 939.1 in normoxia and hypoxia respectively. In arterial SMC, the values were 487.2 and 700.6 in normoxia and hypoxia respectively. The receptor density in venous SMC was significantly higher than in arterial SMC in normoxia and hypoxia. The higher PAF receptor density in venous SMC coupled with the greater binding affinity, will make more receptors available for binding in venous SMC than in arterial SMC, thereby providing congenial pulmonary vascular environment for fetal lungs *in vivo*. We did not

study effect of PAFR antagonist on these binding isotherms, but we have reported that a specific PAFR antagonist displaces PAFR binding to lung membranes in a concentration dependent manner, nullifying the differences in $B_{max}$ and $K_D$ in hypoxia and normoxia[23]. This demonstrates that PAF binds to its specific receptor to evoke a response which is an important requirement for a receptor-mediated event[33]. Even though the characteristics of PAF binding to its receptors may be similar in various tissues, different binding features have been reported in some tissues and cells, under different conditions [40, 41, 42]. For instance, in human platelets, the $K_D$ of PAF binding to platelet membrane receptors varied from 0.05 to 37 nM. In asthmatics, a $K_D$ of 0.38 nM was reported compared with 0.26 nM in nonasthmatic volunteers that have different receptor density[43]. These reports show that PAF binding affinity to its receptors and the $B_{max}$ can be regulated by physiological and pathological conditions. Our findings of different PAF binding affinity to its receptor in arterial SMC and venous SMC, and in normoxia and hypoxia validates a novel control mechanism of PAF receptor binding and suggests an important regulatory mechanism of PAF effects *in vivo*. Therefore, the combination of a downregulation of PAFR $B_{max}$ and the decrease in binding affinity in arterial SMC and venous SMC from hypoxia to normoxia, or perhaps in subcellular cell fractions, will result in a decrease in pulmonary vascular resistance during physiological or pathological hypoxia.

**PAF receptor binding to subcellular SMC fractions**

Physiological and pathological effects of PAF are mediated by its specific G protein coupled receptor [25, 44]. We have shown in previous reports that PAF induces expression of its receptors *in vivo* and *in vitro*, suggesting that it can activate intracellular molecules that regulate gene expression, cell growth, pharmacological, and physiological responses[6, 21,45]. In this report, we used pulmonary vascular SMC to show a common observation of PAFR binding in arterial and venous SMC in pari passu. Previously, it has been shown that nuclear factor-kappa B p65 (NF-kB p65), cyclin dependent kinases, and rho kinase are downstream effectors of PAF-induced PVSMC proliferation, with NF-kB p65 presenting an important link between cytosolic and nuclear responses following PAF stimulation [45, 46, 47, 49, 50]. The foregoing discussion portends that PAF activation of its receptor induces intracellular signaling pathways that result in pulmonary vascular SMC growth and responses, and that interruption of this intracellular signaling by either an endogenous or exogenous molecule, should interfere with normal PAF-mediated reactions. We employed pharmacological manipulations in an *in vitro* setting in normoxia and hypoxia to examine a possible involvement of Rho kinase in PAF-induced pulmonary vascular smooth muscle responses. One major finding in this study is that the protein synthesis inhibitor, cycloheximide, attenuated PAFR binding in membrane, soluble and nuclear fractions of both arterial SMC and venous SMC in normoxia and hypoxia, comparable to the inhibitory effects of CV-3988, a specific PAF receptor antagonist.

**Cycloheximide:** Cycloheximide inhibits protein synthesis by interfering with the translocation of tRNA during protein synthesis in the ribosomes. Cycloheximide has been used to study rate of protein synthesis in rat liver where it was found that it inhibited total cellular protein synthesis including that of mitochondria after a long term treatment[51] and in the hippocampus[51], where it decreased protein synthesis in pyramidal neurons. Our finding of cycloheximide's inhibition of PAFR binding in membrane, soluble and nuclear fractions indicate that the inhibition of binding by cycloheximide is due primarily to suppressed protein synthesis,

including synthesis of PAF receptor protein, in these cells during normoxia and hypoxia. Cycloheximide has also been used to probe physiological response of stimuli in the nucleus in an attempt to define site of such response [45, 52, 53, 54, 55]. In this study, it is seen that cycloheximide is active in decreasing PAFR binding in nuclear fraction just like the specific PAF receptor antagonist CV-3988. This offers a handle for scientists of PAFR-mediated effects to accurately define PAF effects in cytosol and nucleus after purifying these subcellular proteins and isolating the resident organelles, such as mitochondria, in the soluble fraction and nucleolus in the nucleus. Thus PAF receptor activation, desensitization, re-sensitization, and internalization can be quantified and localized following a stimulus[56, 57, 58, 59, 60]. An ability to elucidate these phenomena will greatly enhance our understanding of the mechanism of actions of an exogenous or endogenous stimuli following PAF receptor-linked responses.

**PAF receptor binding and Rho kinase activity**

We have previously shown that: a) smooth muscle cells from pulmonary veins proliferate more than cells from pulmonary arteries in normoxia and in hypoxia and that stimulation of the cells with PAF augments cell proliferation in both conditions, in agreement with our previous reports[16, 45]. Another major finding in this study is that Rho kinase inhibitor, Y-27632 inhibited PAFR binding in membrane, soluble and nuclear fractions of both arterial and venous SMC in normoxia and hypoxia, comparable to the inhibitory effects of cycloheximide, a protein synthesis inhibitor, and CV-3988, a specific PAF receptor antagonist. In a previous study, we reported the involvement Rho kinase in PAF receptor-mediated cell proliferation suggesting that Rho kinase inhibitor Y-27632 may inhibit PAF receptor protein expression resulting in decreased PAFR binding. In that study, whether by cell counting or by DNA synthesis, Rho kinase inhibition by Y-27632 led to a decrease in cell proliferation of both arterial and venous SMC[16]. We also showed that Rho kinase inhibitors Y-27632 and HA-1077 blunted PAF receptor protein expression by both arterial and venous SMC[16]. In the present study, the decrease in PAFR binding to the subcellular fractions of arterial and venous SMC is likely due to inhibition of PAFR protein expression emanating from inhibition of Rho kinase by its inhibitors implying that PAFR binding is decreased because inhibition of Rho kinase prevented PAFR protein expression so as to cause downstream signal transduction. The small GTPase, RhoA, and its effector protein, Rho kinase, are important regulators of vascular reactivity[13, 16, 19, 41, 54, 55, 56, 61, 62, 63]. Inhibition of Rho kinase with Y-27632 resulted in pulmonary vasodilation of ovine fetus even in the presence of Nitric Oxide[18]. Our findings in ovine fetal smooth muscle cells, presented in this report, will suggest that one of the reasons for vasodilation following Rho kinase inhibition with Y-27632 may, in part, reside in the inhibition of PAFR expression, PAFR binding and PAFR-mediated responses. In ovine fetus, in vivo, PAF maintains a high pulmonary vasomotor tone in the hypoxic lung environment[3] and inhibition of the PAFR-mediated effect resulted in decreased pulmonary artery pressure with concomitant increase in pulmonary blood flow. There is significant PAFR in SF and NF proteins from the Y-27632 treated cells, indicating that effect of Rho kinase on PAFR-mediated signaling transcends its effect at the membrane receptor. Effect in cytosolic fraction is logical since PAFR binding in the cytosolic domain of PAF receptor has not been described[63, 64]. A disruption of this congenial interaction between membrane and cytosolic domain of PAF receptors, will result in adverse fetal pulmonary circulatory conditions.

**PAF receptor binding and contractile protein expression**

**Myosin Light Chain Kinase:** Myosin light chain kinase (MLCK) is a protein kinase which phosphorylates the regulatory light chain of myosin II (MLCK2), which is said to be prevalent in smooth muscle[65, 66, 67]. Rho kinase and Myosin regulatory light chain (LC20) phosphorylation play an important role in vascular smooth muscle contraction and cell migration. Ca2+/calmodulin-dependent myosin light chain kinase (MLCK) phosphorylates LC20 which is its only known substrate[67, 68]. In response to contractile stimuli, the small GTPase, RhoA activates its downstream effector Rho kinase which, in turn, promotes contraction via myosin light chain phosphatase inhibition[69]. Consequently Rho kinase modulates MLCK activity and acts in harmony with PAFR activation. We found that both Rho kinase inhibitors Y-27632 and HA-1077 stimulated MLCK2 expression in arterial and venous SMC in normoxia and hypoxia, implicating Rho kinase as important mediator of pulmonary SMC contractility. This is a novel information regarding the involvement of MLCK2 protein expression and Rho kinase activity in pulmonary vascular SMC of the fetus. Of note is the finding that in venous SMC, MLCK2 expression was significantly higher under unstimulated conditions. The relevance of this finding in Rho kinase-MLCK2 activity in fetal pulmonary hemodynamics *in vivo* is yet to be determined, but may explain the need for the pulmonary veins of the fetus to be in constricted state.

**Calponin:** Calponin is a troponin-T like protein that has been implicated in the regulation of smooth muscle contraction through its interaction with F-actin. It binds to actin, myosin, and $Ca^{2+}$-binding proteins, among others[70]. Also RhoA-Rho kinase signaling pathway, in conjunction with Calponin participate in controlling cytoskeletal reorganization[71]. The switch of vascular SMC from the contractile phenotype to proliferative type can contribute to atherosclerosis and neo-intima formation. It was reported that teniposide, a pharmacologic agent, regulates switching of SMC phenotype by upregulating expression of contractile genes in a miR-21-dependent manner, an important anti-atherogenic function[72]**.** We report here, for the first time, significant upregulation of Calponin protein in arterial SMC following treatment with the Rho kinase inhibitors Y-27632 and HA-1077, with both inhibitors producing commensurate upregulation in expression in normoxia or hypoxia. PAF treatment also upregulated Calponin expression. This observation is in line with the report that Rho kinase acts in conjunction with Calponin to regulate cytoskeletal reorganization[72]**.** In venous SMC, profile of Calponin expression is not clearly defined as in arterial SMC. Calponin expression increased with Y-27632 in normoxia, but not in hypoxia, and PAF treatment decreased Calponin expression in normoxia, but increased expression in hypoxia. The consequences of a clearer profile of Calponin expression in arterial but not in venous cells may reside in the need for pulmonary arterial environment of the fetus to be less subject to phenotypic changes than venous environment in order to accommodate the maternal-fetal hemodynamics. Regardless of the novel nature of this finding, the contention we thus propose needs further examination.

**PAF receptor gene expression and summary of findings.**

It has been shown that PAF receptor protein expression is higher in venous than arterial SMC both in normoxia and hypoxia[16, 23, 73]**?,** Also in these reports, PAF treatment of cells in normoxia or hypoxia upregulated PAFR protein expression in both cell types, more so in venous than arterial SMC. Study of PAF receptor gene expression by PA-SMC and PV-SMC under

baseline unstimulated conditions showed greater PAF receptor gene expression in hypoxia for both PA and PV cells, but there was no difference in expression by each cell type in normoxia[74]. PAF receptor gene expression by whole lungs of fetal lambs, akin to hypoxia, was greater than by newborn lambs <1 day old analogous to normoxia[23]. The receptor gene expression data from fetal and <1 day old newborn lamb lungs corroborate our data from direct radioligand binding assays which showed high PAFR binding to SMC in hypoxia, implying that the decrease in PAFR binding in normoxia is due, in part, to downregulation of PAFR gene expression in normoxia. In 6-12 day old newborn lamb arterial SMC, PAF treatment significantly upregulated PAFR gene expression compared to control condition in normoxia, hypoxia or hyperoxia. PAFR gene expression in the newborn venous SMC was not reported[8]. In the current report, PAFR gene expression in venous SMC in control conditions in normoxia is greater than expression by arterial SMC under same conditions. PAF treatment in normoxia produced no difference in expression, while effect of CV-3988 mirrors expression in control condition in normoxia, but quantitatively greater. Hypoxia stimulated quantitatively similar PAFR gene expression under all conditions. These are seemingly paradoxical effect for it is anticipated that more receptor gene expression should translate to more receptor protein, and that CV-3988 should decrease PAFR gene expression. This should not be the case since CV-3988 is protein receptor antagonist and not a protein receptor translational antagonist.

**Summary and future directions**

The present report demonstrates that PAF binding to its receptors in membrane proteins does not preclude binding to other subcellular protein fractions. Although quantitatively lower than membrane protein binding, this information offers the interested researcher the motivation to isolate, purify and characterize the consequences, if any, of PAF binding to subcellular organelles such as mitochondria and ribosomes. The involvement of MLCK2 protein expression and Rho kinase activity in pulmonary vascular SMC of the fetus lends an insight in a mechanism PAF-linked pulmonary vascular contraction, the PAF receptor antagonist, CV-3988, upregulated PAF receptor expression under both condition, an unexpected result that could be addressed by including a protein synthesis inhibitor such as cycloheximide in a similar study. Apart from inhibiting PAF binding to its receptor, an initial step in action of a PAF receptor antagonist such as CV-3988, a highly lipophilic molecule, may entail some conformational disorganization of the receptor protein. This contention will need further investigation of PAF receptor gene expression with other types PAF receptor antagonist, such as WEB 2170. In addition, it will be necessary to explore the involvement of Rho kinase in PAF receptor gene expression by examining the influence of PAF receptor gene expression in the presence of a Rho kinase inhibitor such as Y-27632. This is an important information that is missing from this report.

**Acknowledgements**

This work was supported in part by grants 513292 by the Lundquist Institute, Torrance, CA

**References**


1. Argiolas L, Fabi F, del Basso P. Mechanisms of pulmonary vasoconstriction and bronchoconstriction produced by PAF in guinea-pig: role of platelets and cyclo-oxygenase metabolites. *Br J Pharmacol.* 1995;114: 203-209. doi: 10.1111/j.1476-5381.1995.tb14926.x. PMID: 7712019
2. 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. doi: 10.1152/ajpheart.1995.269.2.H704.PMID: 7653635
3. Ibe BO, Hibler S, Raj JU. Platelet-activating factor modulates pulmonary vasomotor tone in the perinatal lamb. *J Appl Physiol*. 1998;85: 1079-1085. DOI: 10.1152/jappl.1998.85.3.1079. PMID: 9729586.
4. Raj JU, Toga H, Ibe BO, Anderson J. Effects of endothelin, platelet activating factor and thromboxane $A_2$ in ferret lungs. *Respir Physiol.* 1992;88: 129-140. doi: 10.1016/0034-5687(92)90034-t. PMID: 1626132
5. Toga H, Hibler S, Ibe BO, Raj JU. Vascular effects of platelet-activating factor in lambs: role of cyclo- and lipoxygenase. *J Appl Physiol.* 1992;73: 2559-2566. doi: 10.1152/jappl.1992.73.6.2559. PMID: 1490969
6. 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. Doi: 10.1152/ajplung.00089.2007.
7. Ono S, Westcott JY, and Voelkel NF. PAF antagonists inhibit pulmonary vascular remodeling induced by hypobaric hypoxia in rats. *J Appl Physiol*. 1992;73: 1084-1092. doi: 10.1152/jappl.1992.73.3.1084. PMID: 1400021
8. Hanouni M, Bernal G, Shaemion S, Narvaez VRF, Ibe BO. Hypoxia and hyperoxia potentiate PAF receptor-mediated effects in newborn ovine pulmonary arterial smooth muscle cells: significance in oxygen therapy of PPHN Physiol Rep. 2016; 4: 1-18. doi: 10.14814/phy2.1284. PMID: 27354543
9. Gao Y, Raj JU. Regulation of the pulmonary circulation in the fetus and newborn. *Physiol Rev* 90: 1291-1335, 2010. doi: 10.1152/physrev.00032.2009. PMID: 20959617
10. Parent J-L, 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. doi: 10.1074/jbc.271.38.23298. PMID: 8798529
11. Witzenrath M, Gitbier B, Owen JS, Schmeck B, Mitchell TJ, Mayer K, Thomas MJ, Ishii S, Rosseau S, Suttorp N Schutte H. Role of platelet-activating factor in pneumolysin-induced acute lung injury. *Crit Care Med*. 2007;7: 1756-1762. doi: 10.1097/01.CCM.0000269212.84709.23. PMID: 17522574
12. Madden JA, Dantuma MW, Sorokin EA, Weihrauch D, Kleinman JG. Telokin expression and the effect of hypoxia on its phosphorylation status in smooth muscle cells from small and large pulmonary arteries. Am Jo Physiol lung Cell Mol Physiol. 2008;294, L1166-L1173. doi 10.1152/ajplung.00375.2007. PMID:
13. Martin C, Göggle R, Ressmeyer A-R, Uhlig S. Pressor responses to platelet-activating factor and thromboxane are mediated by Rho-kinase. *Am J Physiol Lung Cell Mol Physiol*. 2004;287: L250-L257.

DOI: 10.1152/ajplung.00420.2003
PMID: 15064228

14. Etienne-Manneville S, Hall A. Rho GTPases in cell biology. *Nature* 2002;Dec 12;420: 629-635. doi: 10.1038/nature01148. PMID: 12478284.
15. Ward JPT, McMurtry IF. Mechanisms of hypoxic pulmonary vasoconstriction and their roles in pulmonary hypertension: new findings for an old problem. *Curr Opin Pharmacol*. 2009, June;9: 287-296. doi: 10.1016/j.coph.2009.02.006. PMID: 19297247
16. Renteria LS, Austin M, Lazaro M, Andrews MA, Lustina J, Raj JU, Ibe B. RhoA- Rho kinase and platelet-activating factor stimulation of ovine foetal pulmonary vascular vascular smooth muscle cell proliferation. Cell Prolif. 2013;46: 563-575. Doi: 10.1111/cpe.12052, PMID: 24033386.
17. Beqaj S, Jakkaraju S, Mattingly RR, Pan Desi, Schuger L. High RhoA activity maintains the undifferentiated mesenchymal cell phenotype, whereas RhoA down-regulation by laminin-2 induces smooth muscle myogenesis. *J Cell Biol*. 2002 Mar 4;156: 893-903. doi: 10.1083/jcb.200107049. Epub 2002 Mar 4. PMID: 11877460
18. Alvira CM, Sukovich DJ, Lyu S-C, Cornfield DN. Rho kinase modulates postnatal adaptation of the pulmonary circulation through separate effects on pulmonary artery and smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol*. 2010;299: L872-L878. doi: 10.1152/ajplung.00199.2010. PMID: 20709731
19. Fagan KA, Oka M, Bauer NR, Gebb SA, Ivy DD, Morris KG, McMurtry IF. Attenuation of acute hypoxic pulmonary vasoconstriction and hypoxic pulmonary hypertension in mice by inhibition of Rho-kinase. *Am J Physiol Lung Cell Mol Physiol.* 2004;287: L656-664. doi: 10.1152/ajpheart.00394.2002. PMID: 14977625
20. Patil SB, Bitar KN. RhoA- and PKC-α-mediated phosphorylation of MYPT and its association with HSP27 in colonic smooth muscle cells. *Am J Physiol Gastrointest Liver Physiol*. 2006 Jan;290: G83-95. doi: 10.1152/ajpgi.00178.2005. Epub 2005 Sep 22. PMID: 16179599
21. Ibe BO, Portugal AM, Chaturvedi S, Raj JU. Oxygen-dependent PAF receptor binding and intracellular signaling in ovine fatal pulmonary vascular smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2005; 288:L879-886. Doi: 10.1152/ajplung.00341.2004 PMID: 15618453.
22. Ibe BO, Portugal AM, Raj JU. Metabolism of platelet activating factor by intrapulmonary vascular smooth cells. Effect of oxygen on phospholipase $A_2$ protein expression and activities of acetyl-CoA acetyltransferase and cholinephosphotransferase. *Mol Genet Metab.* 2002 Nov;77: 237-248. doi: 10.1016/s1096-7192(02)00147-6. PMID: 12409272
23. Ibe BO, Sander FC, Raj JU. Platelet-activating factor receptors in lamb lungs are downregulated immediately after birth. *Am J Physiol Heart Circ Physiol.* 2000;278: H1168-H1176. Doi: 10. 1152/ajpheart.2000.278-4.H1168. PMID: 10749711.
24. Braquet P, Rola-Pleszczynski M. The role of PAF in immunological responses: a review. *Prostaglandins.* 1987 Aug;34(2):143-148. doi: 10.1016/0090-6980(87)90190-0. PMID: 3313527
25. Dupré DJ, Rola-Pleszczynski M, Stankova J. Rescue of internalization-defective platelet-activating factor receptor function by EBP50/NHERF1. *J Cell Commun Signal.* 2012; 6:205-215. Doi 10. 1007/s12079-012-0175-1. PMID:

22878922.
26. Zimmerman B, Simaan M, Lee M-H, Luttrell LM, Laporte SA. C-Src-mediated phosphorylation of AP-2 reveals a general mechanism for receptors internalizing through the clathrin pathway. Cell Signal. 2009;21: 103-110. Doi: 10. 1016/j.cellig.2008.09.013 PMID: 18938240.
27. Bhosle VK, Rivera JC, Zhou T (Ellen), Omri S, Sanchez M, Hamel D, Zhu T, Rouget R, Al Rabea A, Hou X, Lahaie I, Ribeiro-da-Silva A, Chemtob S. Nuclear localization of platelet-activating factor receptor controls retinal neovascularization. *Cell Discovery*. 2016;2: 16017. Doi: 10.1038/celldisc.2016.17. PMID: 27462464
28. Jobe AH, Polk DH, Ervin MG, Padbury JF, Rebello, CM, Ikegami M. Preterm betamethasone treatment of fetal sheep: outcome after term delivery. *J Soc Gynecol Investig.* 1996;3(5): 250-258. Doi: 10.1016/s1071-5576(96)00029-9. PMID: 8796837.
29. Bland RD. Neonatal chronic lung disease in post-surfactant era. *Biol Neonate*. 2005;88(3): 181-191. Doi: 10.1159/000087581. PMID: 16210840.
30. Sehgal A, Allison BJ, Miller SL, Polglase GR, McNamara PJ, Hooper SB. Impact of acute and chronic hypoxia-ischemia on the transitional circulation. *Pediatrics.* 2021; 147(3):e2020016972. Doi: 10.1542/peds.2020-016972. PMID: 33622795.
31. Abman SH. Recent advances in the pathogenesis and treatment of persistent pulmonary hypertension of the newborn. *Neonatology.* 2007;94(4): 283-290. Doi: 10. 1159/000101343. PMID: 17575471.
32. Kemp MW, Jobe AH, Usuda H, Nathanielsz PW, Cun L, Kuo A, Huber HF, Clarke GD, Saito M, Newnham JP, Stock SJ. Efficacy and safety of antenatal steroids. *Am J Physiol Regul Integr Comp Physiol.* 2018; 315(4); R825-R839. Doi: 10. 1152/ajpregu.00193.2017. PMID: 29641233.
33. Kemp MW, Saito M, Usuda H, Molloy TJ, Miura Y, Sato Sato S, Watanabe S, Clarke M, Fossler M, Scmidt A, Kallpur SG, Kramer BW, Newnham JP, Jobe AH. Maternofetal pharmacokinetics and fetal lung responses in chronically catheterized sheep receiving constant, low-dose infusions of betamethasone phosphate. *Am J Obstet Gynecol.* 2016 Dec; 215(6): 775.e1-775.e12. doi: 10. 1016/j.ajog.2016.08.017. PMID: 27555319.
34. Schrauben EM, Saini BS, Darby JRT, Soo JY, Lock MC, Stirrat E, Stortz G, Sled JG, Morrison JL, Seed M, Macgowan CK. *J Cardiovasc Magn Reson.* 2019 Jan 21:21(1):8. Doi: 10. 1186/s12968-018-0512-5. PMID: 30661506.
35. Chabrier PE. Growth factors and vascular wall. *Int Angiol*.1996;15: 100-1003. PMID: 8803632
36. McManus LM, Pinckard RN. PAF, a putative mediator of oral inflammation. *Crit Rev Oral Biol Med*. 2000;11: 240-258. doi: 10.1177/10454411000110020701. PMID: 12002818
37. Accurso FJ, Abman SH, Wilkinging RB, Worthen GS, Hanson P. Fetal pulmonary vasodilation after exogenous platelet-activating factor. J Appl Physiol. 1991;70: 778-787. doi: 10.1152/jappl.1991.70.2.778. PMID: 2022570.
38. Burhop KE, Van de Zee H, Bizios R, Kaplan JE, Malik AB. Pulmonary vascular responses to platelet-activating factor in awake sheep and the role of cyclooxygenase metabolites. Am Rev Respir Dis. 1986 Sep;143: 548-554. doi: 10.1164/arrd.1986.134.3.548. PMID: 3752711.
39. Basil O Ibe, Adnan Ameer, Ada Mae Portugal, Lissette Renteria, J Usha Raj.

Platelet-activating factor modulates activity of cyclic nucleotides in fetal ovine pulmonary vascular smooth muscle. J Pharmacol Exp Ther. 2007 Feb;320(2):728-737. doi: 10.1124/jpet.106.111914. PMID: 17085546.

40. Laduron PM. Criteria for receptor sites in binding studies. Biochem Pharmacol. 1984 Mar 15;33(6):897-903. doi: 10.1016/0006-2952(84)90443-x. PMID: 6201175
41. Chao W and Olson MS. Platelet-activating factor: receptors and signal transduction. Biochem J. 1993 Jun 15;292 ( Pt 3)(Pt 3):617-629. doi: 10.1042/bj2920617. PMID: 8391253.
42. Chen J, Ziboh V, and Giri SN. Up-regulation of platelet-activating factor in lung, and alveolar macrophages in bleomycin-hamster model of pulmonary fibrosis. J Pharmacol Exp Ther. 1997 Mar;280(3):1219-1227. PMID: 9067307
43. Burgers JA, Bruynzeel PLB, Mengeler HJJ Kreukniet J, Akkerman J-WN. Occupancy of platelet receptors for platelet activating factor in asthmatic patients during an allergen-induced bronchoconstrictive reaction. J Lipid Mediat. 1993 Jun;7(2): 135-149. PMID: 8400115
44. Chang W, Chen J, Schlueter CF, Hoyle GW. Common pathways for activation of proinflammatory gene expression by G protein-coupled receptors in primary lung epithelial and endothelial cells. *Exp. Lung Res.* 2009;35: 324–343. Doi: PMID: 19415549.
45. 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. DOI: 10.1111/j.1365-2184.2008.00517.x PMID: 18336468.
46. Patil SB, Bitar KN RhoA- and PKC-α-mediated phosphorylation of MYPT and its association with HSP27 in colonic smooth muscle cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* 2006;290: G83–G95. DOI: 10.1152/ajpgi.00178.2005 PMID: 16179599
47. Fagan KA, Oka M, Bauer NR, Gebb SA, Ivy DD, Morris G *et al.* Attenuation of acute hypoxic pulmonary vasoconstriction and hypoxic pulmonary hypertension in mice by inhibition of Rho-kinase**.** *Am. J. Physiol. Lung Cell. Mol. Physiol.* 2004;287: L656–L664. DOI: 10.1152/ajplung.00090.2003. PMID: 14977625.
48. Parker TA, Roe G, Grover TR, Abman SH. Rho kinase activation maintains high pulmonary vascular resistance in the ovine fetal lung**.** *Am. J. Physiol. Lung Cell. Mol. Physiol.* 2006:**291**, L976–L982. DOI: 10.1152/ajplung.00512.2005 PMID: 16815887
49. Carlson SA, Chatterjee TK, Fisher RA (1996) The third intracellular domain of the platelet-activating factor receptor is critical determinant in the receptor coupling to phosphoinositide phospholipase C activating G proteins**.** *J. Biol. Chem.* 1996;272: 23146–23153. DOI: 10.1074/jbc.271.38.23146. PMID: 8798508.
50. Satav J G, Katyare S S, Fatterparker P, Sreenivasan A. Study of protein synthesis in rat liver mitochondria use of cycloheximide. Eur J Biochem. 1977; 73: 287-96. doi: 10.1111/j.1432-1033.1977.tb11318.x. PMID: 837942
51. Gordon RY, Mugantseva EA, Khutzian SS, Podolski IY. Cycloheximide-induced inhibition of protein synthesis in hippocampal pyramidal neurons is time-dependent: differences between CA1 and CA3 areas. Neurosci Lett. 2009;461: 249-251. doi:

10.1016/j.neulet.2009.06.042. PMID: 19545598.

52. Lafarga M, Berciano MT, Andres MA, Testillano PE. Effects of cycloheximide on the structural organization of the nucleolus and the coiled body in normal and stimulated supraoptic neurons of the rat. J Neurocytol. 1994;23: 500-513. doi: 10.1007/BF01184073. PMID: 7983476
53. Lafarga M, Martinez-Guijarro FJ, Berciano MT, Blasco-Ibañez JM, Andres MA, Mellström B, Lopez-Garcia BC, Naranjo JR. Nuclear Fos domains in transcriptionally activated supraoptic nucleus neurons. Neuroscience. 1993;57: 353-564. doi: 10.1016/0306-4522(93)90068-q. PMID: 8115044.
54. Agrawal V, Jaiswal MK, Ilievski V, Beaman KD, Jilling T, Hirsch E. Platelet-activating factor: a role in preterm delivery and an essential interaction with Toll-like receptor signaling in mice. Biol Reprod. 2014;91: 119. doi: 10.1095/biolreprod.113.116012. PMID: 25253732
55. V Boulom, H-W Lee, L Zhao, M Eghbali-Webb. Stimulation of DNA synthesis, activation of mitogen-activated protein kinase ERK2 and nuclear accumulation of c-fos in human aortic smooth muscle cells by ketamine. Cell Prolif. 2002;35: 155-165. doi: 10.1046/j.1365-2184.2002.00233.x. PMID: 12027951.
56. Gupta MK, Mohan ML, Prasad SVN. G-protein-coupled receptor resensitization paradigms. Int Rev Cell Mol Biol. 2018;339: 63-91. Doi: 10.1016/bs.rcmb.2018.03.002. PMID: 29776605.
57. Kliewer A, Reinscheid RK, Schulz S. Emerging Paradigms of G Protein-Coupled Receptor Dephosphorylation. Trends Pharmacol Sci. 2017;38: 621-636. doi: 10.1016/j.tips.2017.04.002. PMID: 28478994.
58. Calebiro D, Godbole A. Internalization of G-protein-coupled receptors: Implication in receptor function, physiology and diseases. Best Pract Res Clin Endocrinol Metab. 2018;32: 83-91. doi: 10.1016/j.beem.2018.01.004. PMID: 29678288.
59. Weis W, Kobilka BK. The molecular basis of G protein-coupled receptor activation. Annu Rev Biochem. 2018; 87: 897-919. Doi: 10.1146/annurev-biochem-060614-033910. PMID: 29925258.
60. Nagaoka T, Fagan KA, Gebb SA, Morris KG, Suzuki T, Shimokawa H, McMurtry IF, Oka M. Inhaled Rho kinase inhibitors are potent and selective vasodilators in rat pulmonary hypertension. *Am J Respir Crit Care Med.* 2005;171: 494-499. DOI: 10.1164/rccm.200405-637OC. PMID: 15563635.
61. Somlyo AP, Wu X, Walker LA Somlyo AV. Pharmacomechanical coupling: the role of calcium, G-proteins, kinases and phosphatases. *Rev Physiol Biochem Pharmacol* 1999;130: 201-234. doi: 10.1007/3-540-64753-8_5. PMID: 10087910.
62. Badejo AM, Dhaliwal JS, Casey DB, Gallen TB, Greco AJ, Kadowitz PJ. Analysis of pulmonary vasodilator responses to the Rho-kinase inhibitor fasudil in the anesthetized rat. *Am J Physiol Lung Cell Mol Physiol* 2008;295: L828-L836. doi: 10.1152/ajplung.00042.2008. Epub 2008 PMID: 18689606.
63. Le Gouill C, Parent JL, Rola-Pleszczynski M, Stanková J. Structural and functional requirements for agonist-induced internalization of the human platelet-activating factor receptor. J Biol Chem. 1997;272: 21289-21295. doi: 10.1074/jbc.272.34.21289. PMID: 9261140.
64. Gao Y, Ye LH, Kishi H, Okagaki T,

Samizo K, Nakamura A, Kohama K. Myosin light chain kinase as a multifunctional regulatory protein of smooth muscle contraction IUBMB Life. 2001;51:337-344. doi: 10.1080/152165401753366087 PMID:11758800.

65. Wang L, Jia C, Yu Z, Liu X, Kang L, Cong Y, Shan Y, Zhao Z, Ma B, Cong Y. Pennogenin tetraglycoside induces rat myometrial contraction and MLC20 phosphorylation via PLC-IP(3) and RhoA/Rho kinase signaling pathways. PLoS One. 2012;7 :e51536. doi: 10.1371/journal.pone.0051536. PMID: PMID: 23251567
66. Jin Y, Blikslager AT. The Regulation of Intestinal Mucosal Barrier by Myosin Light Chain Kinase/Rho Kinases. Int J Mol Sci. 2020,18;21:3550. doi: 10.3390/ijms21103550. PMID: 3244341.
67. Ai S, Kuzuya M, Koike T, Asai T, Kanda S, Maeda K, Shibata T, Iguchi A. Rho-Rho kinase is involved in smooth muscle cell migration through myosin light chain phosphorylation-dependent and independent pathways. Atherosclerosis. 2001;155: 321-327. doi: 10.1016/s0021-9150(00)00585-2. PMID: 11254902.
68. Deng JT, Bhaidani S, Sutherland C, MacDonald JA, Walsh MP. Rho-associated kinase and zipper-interacting protein kinase, but not myosin light chain kinase, are involved in the regulation of myosin phosphorylation in serum-stimulated human arterial smooth muscle cells. PLoS One. 2019,13;14:e0226406. doi: 10.1371/journal.pone.0226406. eCollection 2019. PMID: 31834925.
69. Szasz T, Webb RC. Rho-Mancing to Sensitize Calcium Signaling for Contraction in the Vasculature: Role of Rho Kinase. Adv Pharmacol. 2017;78: 303-322.doi: 10.1016/bs.apha.2016.09.001. PMID: 28212799.
70. Lu J, Landerholm TE, Wei JS, Dong XR, Wu SP, Liu X, Nagata K, Inagaki M, Majesky MW. Coronary smooth muscle differentiation from proepicardial cells requires rhoA-mediated actin reorganization and p160 rho-kinase activity. Dev Biol. 2001,15;240: 404-418. doi: 10.1006/dbio. 2001.0403. PMID: 11784072
71. Hellstrand P, Albinsson S. Stretch-dependent growth and differentiation in vascular smooth muscle: role of the actin cytoskeleton. Can J Physiol Pharmacol. 2005;83:869-875. doi: 10.1139/y05-061. PMID: 16333359
72. Han H, Yang S, Liang Y, Zeng P, Liu L, Yang X, Duan Y, Han J, Chen Y. Teniposide regulates the phenotype switching of vascular smooth muscle cells in a miR-21-dependent manner. Biochem Biophys Res Commun. 2018;506: 1040-1046. doi: 10.1016/j.bbrc.2018.10.198. PMID: 30409428.
73. Renteria LS, Raj JU, Ibe BO. Prolonged hypoxia modulates platelet activating factor receptor mediated responses by fetal ovine pulmonary vascular smooth muscle cells. Mol Genet Metab. 2010;101: 400-404. Doi:10. 1016/j-ymgme.2010.08.005. PMID:
74. Renteria LS, Cruz E, Ibe BO. Platelet-activating factor synthesis and receptor-mediated signaling are downregulated in ovine newborn lungs: relevance in postnatal pulmonary adaptation and persistent pulmonary hypertension of the newborn. J Dev Orig Health Dis. 2013 Dec;4(6):458-469. doi: 10.1017/S2040174413000366. PMID: 24924229.