Molecular Genetics and Metabolism Reports 4 (2015) 11–18

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism Reports

journal homepage: http://www.journals.elsevier.com/molecular-genetics-and-metabolism-reports/

Mechanism by which nuclear factor-kappa beta (NF-kB) regulates ovine fetal pulmonary vascular smooth muscle cell proliferation

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ARTICLE INFO

Available online 3 June 2015

Keywords:
PAF receptor
NF-kB p65 signaling retinoblastoma protein
DNA binding

ABSTRACT

Platelet activating factor (PAF) modulates ovine fetal pulmonary hemodynamic. PAF acts through its receptors (PAFR) in pulmonary vascular smooth muscle cells (PVSMC) to phosphorylate and induce nuclear translocation of NF-kB p65 leading to PVSMC proliferation. However, the interaction of NF-kB p65 and PAF in the nuclear domain to effect PVSMC cell growth is not clearly defined. We used siRNA-dependent translation initiation arrest to study a mechanism by which NF-kB p65 regulates PAF stimulation of PVSMC proliferation. Our hypotheses are: (a) PAF induces NF-kB p65 DNA binding and (b) NF-kB p65 siRNA attenuates PAF stimulation of PVSMC proliferation. For DNA binding, cells were fed 10 nM PAF with and without PAFR antagonists WEB 2170, CV 3988 or BN 52021 and incubated for 12 h. DNA binding was measured by specific ELISA. For NF-kB p65 siRNA effect, starved cells transfected with the siRNA were incubated for 24 h with and without 10 nM PAF. Cell proliferation was measured by DNA synthesis while expression of NF-kB p65 and PAFR protein was measured by Western blotting. In both studies, the effect of 10% FBS alone was used as the positive control. In general, PAF stimulated DNA binding which was inhibited by PAFR antagonists. siRNAs to NF-kB p65 and PAFR significantly attenuated cell proliferation compared to 10% FBS and PAF effect. Inclusion of PAF in siRNA-treated cells did not reverse inhibitory effect of NF-kB p65 siRNA on DNA synthesis. PAFR expression was inhibited in siRNA-treated cells. These data show that PAF-stimulation of PVSMC proliferation occurs via a PAFR-NF-kB p65 linked pathway.

1. Introduction

Platelet activating factor (PAF) is an endogenous phospholipid with a diverse range of physiological and pathological activities, including vascular reactivity, aggregation of platelets, glycogen degradation, reproduction, brain function, blood circulation, and as a mediator of inflammation [1–3]. But it is primarily a mediator of intracellular interactions [4]. PAF is produced by a variety of cells including smooth muscle cells, endothelial cells, neutrophils, monocytes and macrophages. However, inflammatory cells produce PAF in much greater quantities when required in response to cell-specific stimuli [1,2,5–9]. In fetuses, PAF maintains a high vasomotor tone necessary for pulmonary circulation. In the newborn, however, increased levels of PAF in the pulmonary circulation can result in persistent pulmonary hypertension of the newborn (PPHN) as a result of excessive vasoconstriction [10]. In the pulmonary vasculature, cAMP and cGMP concentrations are regulated in part by a specific cyclic nucleotide dependent phosphodiesterase (PDE), such as PDE5, and other mediators of pulmonary vascular reactivity which modulate perinatal pulmonary hemodynamics [10]. Nuclear factor-kappa beta p65 (NF-kB p65) is one of the downstream regulators of PAF mediated signaling in fetal ovine pulmonary vascular smooth muscle cells (PVSMC), where it has been shown to activate cyclin dependent kinases (cdk) 2 and 4 leading to cell proliferation [11]. NF-kB is a family of transcription factors that modulate DNA transcription. It plays a key role in regulating immune response to infection and the inflammatory response [12,13]. As a primary transcription factor during inflammatory processes, NF-kB acts as first responder to cell stimulation by cytokines such as TNF and LPS and PAF leading to fairly rapid changes in target gene expression [14,15]. Also, following an appropriate stimulus NF-kB is activated via phosphorylation and proteosome dependent degradation of cytosolic IkBα [12,13]. Activated NF-kB p65 is then translocated into the nucleus [12–13,16]. NF-kB p65 is a member of the NF-kB family of transcription factors which is activated by PAF receptor mediated signaling. Under normal physiological conditions PAF is minimally produced, however it is abundantly produced under inflammatory conditions associated with tissue injury [17,18]. PAF also stimulates proliferation of SMC of systemic origin [9,19–21] via pathway involving its G-protein-coupled receptor. For instance, PAF stimulates growth of aortic SMCs [21] and human bronchial SMCs in culture [22]. We have reported that PAF stimulates phosphorylation and nuclear translocation of NF-kB p65 resulting in pulmonary vascular smooth muscle cell proliferation [11]. Persistent pulmonary hypertension of the newborn is characterized by elevated pulmonary vascular resistance and pressure due to vascular remodeling and increased vessel

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http://dx.doi.org/10.1016/j.ymgmr.2015.05.003

tension secondary to chronic hypoxia during the fetal and newborn period [23,24]. In comparison to the adult, the pulmonary vasculature of the fetus and the newborn undergoes tremendous developmental changes that increase susceptibility to a hypoxic insult [23]. Substantial evidence indicates that chronic hypoxia alters the production and responsiveness of various vasoactive agents such as endothelium-derived nitric oxide, endothelin-1, prostanoids and PAF resulting in sustained vasoconstriction and vascular remodeling [25]. These changes occur in most cell types within the vascular wall, particularly endothelial and smooth muscle cells that appear to be critical to the development of hypoxic pulmonary hypertension of the newborn [25,26]. Following our previous report on the involvement of NF-kB p65 in PAF stimulation of pulmonary vascular smooth muscle cell proliferation [11], we wished to further investigate the intracellular mechanisms by which PAF acts through its receptor to stimulate cell proliferation through NF-kB p65 to induce gene expression and cell growth. Furthermore, our previous report concentrated on involvement of the MAPK pathway in NF-kB p65 activation. In this report our goal is to further explore the mechanism by which PAF receptor activation induces nuclear translocation of NF-kB p65 resulting in stimulation of proliferation of ovine fetal PVSMC. Our primary hypothesis is that PAF stimulates expression and phosphorylation of IkBα, the upstream signal for NF-kB p65 nuclear translocation, which induces expression of retinoblastoma (Rb) protein leading to gene expression and cell proliferation.

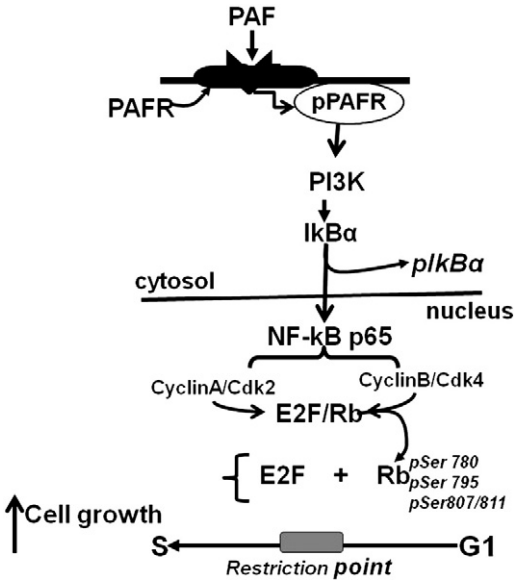

Fig. 1. Scheme of study hypothesis.

2. Materials and methods

2.1. Materials

The studies were approved by the Institutional Animal Care and Use Committee of Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center. Pregnant ewes (146–148 day gestation, term being 150 days) 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) as well as NF-kB p65 were purchased from Biomol Research Plymouth Meeting, PA. ^{3}H-thymidine was purchased from Perkin Elmer Life Sciences (Boston, MA). Phenylmethysulfonyl fluoride (PMSF), leupeptin, pepstatin, bovine serum albumin (BSA), as well as antibody to actin were purchased from Sigma-Aldrich Company (St. Louis, MO). Antibody to PAFR 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 or as indicated by the respective study reagents.

2.2. Methods

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

Intrapulmonary vessels were isolated from freshly killed term fetal lambs and then smooth muscle cells were harvested from the freshly excised arteries under sterile conditions as previously reported [26–27]. Cells were used at the 4th to 10th passages and the identity of the smooth muscle cells at each passage was characterized with a smooth muscle cell-specific monoclonal antibody, (Sigma-Aldrich, St. Louis, MO). The SMC were devoid of endothelial cells and fibroblasts. Cell synthetic and proliferative phenotype did not change from 4th to 10th passages as has been shown in our previous reports [26].

2.3. Study designs

Fig. 1 shows our study hypothesis conjectured from our previous publications [11,27]. PAF binding to its G_q G protein coupled receptor leads to activation of the receptor by phosphorylation (pPAFR). Subsequent intracellular signaling processes result in the phosphorylation and ubiquinization of IkBα (pIkBα), which results in the nuclear translocation of NF-kB p65. In its nuclear domain, NF-kB p65 activates cyclin A and cyclin B to synthesize Cdk2 and Cdk4 respectively [11]. This results in uncoupling of EF2/Rb dimer. CdK2/4 phosphorylate retinoblastoma (Rb) protein at specific serine residues, which activates cell cycle from the G1 phase to S phase leading to DNA replication and cell growth. The involvement of PAFR-mediated signaling and NF-kB p65 activation in proliferation of ovine fetal pulmonary vascular smooth muscle cells will be studied and expression of specific proteins: PAFR, IkBα, Cdk2, and Rb protein will be measured.

2.4. Study conditions

All studies were done in vitro on smooth muscle cells from intrapulmonary arteries (PASMC). Adherent cells were cultured in normoxia according to the specific experimental protocol.

2.4.1. Normoxia

Cells were studied in humidified incubator at 37 °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.

2.4.2. Proliferation assay

Proliferation assays were standardized as we previously reported [11]. Briefly, cells were seeded in 6-well culture plates at 5×10^4 cells per well and allowed to stabilize for 2–3 days. The cells were then serum-starved by culturing in 0.1% FBS for 72 h, then cells were cultured in 10% FBS with or without the test agents in the presence of 5 μCi/well of ^{3}H-thymidine and incubated for 24 h more in normoxia according to the specific protocol. We elected to do all our subsequent studies by incubating the cells for 24 h after the 72 h starvation with 10% FBS and assaying for DNA with 5% trichloroacetic acid followed with 0.5 N sodium hydroxide (NaOH). The test agents were dissolved in 10% FBS, which was also used as the control in all culture conditions. After 24 h treatment, the culture plates were placed on ice and the culture medium was aspirated and cells were washed with ice-cold PBS, followed with wash with ice-cold 5% trichloroacetic acid. Then cells labeled with ^{3}H-thymidine were extracted with 0.5 N NaOH. Radioactivity of

cell lysate was quantified on an LKB 6500 scintillation counter (Beckman Coulter, Fullerton CA). ^{3}H-thymidine was not added to cell proliferation studies quantified by cell counting. The 480 g supernatant was decanted and then the radioactivity present in the supernatant fraction was also determined. The nuclear pellet was extracted with 1 ml PBS. The extract and the wash were combined and transferred to a scintillation vial and then Ecolite scintillation cocktail (MP Biochemicals) was added to the nuclear fraction and the radioactivity was determined using the Beckman liquid scintillation spectrometer. Studies were performed as described above using 10 nM concentrations of PAF as we have determined that this concentration of PAF produced the most reproducible effect on cell growth and protein expression [11,27].

2.5. Specific protocols

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

The effects of inactive PAF metabolite lyso-PAF and PAF receptor antagonist WEB 2170 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 WEB 2170, 1 μM of CV 3988 or with 10% FBS growth media alone, and then 10 nM PAF and 5 μCi of [^{3}H]-thymidine was added to the cells and incubated for 24 h more. The 10% FBS control received neither the WEB 2170 nor the 10 nm lyso-PAF.

2.5.2. Transient cell transfection

Cells were seeded in 6-well culture plates at 5×10^{4} cells per well in an antibiotic-free growth media, and allowed to stabilize for 24 h. Then they were treated with 1.5 μg/ml of each plasmid in lipofectamine transfection reagent with 50 nM of specific siRNA in the PAF signaling pathway of interest. The siRNA were, PAFR siRNA, NF-kB p65 siRNA, and retinoblastoma (Rb) siRNA. Studies were conducted according to the vendors' protocols: PAFR siRNA (Santa Cruz Biotechnology), NF-kB p65 and retinoblastoma (Rb) siRNAs (Cell Signaling) and incubated for 48 h after which the transfection-medium was replaced with fresh 10% FBS culture media, which was used to study cell proliferation or to prepare proteins for Western blotting. Transfection efficiency was between 25 and 35% within 24 h of transfection as judged by the pGFP fluorescence. The proliferative phenotype of transfected cells was compared to untransfected cells. In studying cell proliferation or protein expression, transfected cells were incubated for 24 h, with and without 10 nM PAF in 10% FBS. The control for cell proliferation is 10% FBS alone while cells transfected with scrambled siRNA (Sham siRNA) were used as control for siRNA effect.

2.5.3. DNA binding

DNA binding assays were done with DNA assay kit, TransAM NF-kB p65 DNA binding kit, purchased from Active Motif (Carlsbad, CA, Catalog #40096, and 40596). Assay was performed according to the vendor's protocol. Briefly, cells that were serum starved for 72 h were pulsed for 6 h with 100 nM lyso-PAF, 10 nM PAF, the PAF receptor antagonists: CV 3988, 1.0 μM; WEB 2170, 10 μM; and BN 52021, 100 μM/ml. DNA was extracted from each treatment and NF-kB p65 DNA binding was measured at absorbance of 450 nm with reference at 655 nm. The three types of PAFR antagonist were used to authenticate PAFR-mediated binding to DNA. The control was cells treated with 10% FBS culture media.

2.6. Western blotting

2.6.1. Preparation of proteins for Western analysis

Western blotting was done according to our previous reports. Briefly, after incubation in 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 14,000 *g* for 10 min in refrigerated Ependorff bench centrifuge and stored in 0.2 ml aliquots at −80 °C and used for Western blotting.

2.6.2. SDS-PAGE

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 1–2 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 2 h of SDS-PAGE, proteins were transferred to nitrocellulose membranes by means of Mini Trans-Blot (Bio-Rad, Redmond, CA, USA) at 70 V and then blocked with 5% non-fat dry milk in 1% Tween-20/TBS (T-TBS) overnight. Blots were then incubated with the appropriate dilution of the specific antibody against: for instance, PAFR protein, NF-kB p65 and Rb proteins, after which the gels were washed with 1% T-TBS, incubated for 1 h with an anti-rabbit 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 digitized to quantify blot density. Then, blots were stripped and re-probed for expression of beta actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) which, are constitutively expressed proteins which were used as internal standards.

2.7. Data analysis

For proliferation studies, depending on the specific protocol, cell proliferation is reported as cell number or as cell proliferation in disintegrations per minute (DPM) of measured ^{3}H-thymidine per million cells. All protein expression data are reported as ratio of densitometry of the protein measured to that of beta actin protein standard or that of GAPDH. In all instances where radioisotope was used, background radioactivity was subtracted before quantifying radioactivity. All numerical data are presented as means ± SEM. Data were analyzed with two-tailed t-test followed with ANOVA (GraphPad Prism 6, San Diego, CA). Results were considered significant at $p < 0.05$.

3. Results

3.1. The inactive PAF analogue, lyso-PAF did not stimulate cell proliferation and PAF receptor antagonist WEB 2170 inhibited PAF-induced cell proliferation

Initial quantification of PASMC proliferation by cell counting showed that treatment with 10 nM PAF increased number of cells in the wells. The data means ± SEM, $n = 4$ are as follows. With 10% FBS control, cell count was 15,000 ± 2500 cells/well, which increased to 33,000 ± 3000 cells/well under treatment with 10 nM PAF.

Fig. 2 shows the effect of lyso-PAF and WEB 2170 on proliferation of the PASMC.

Treatment of cells with 10 nM PAF significantly increased cell proliferation compared to the 10% FBS control. Treatment of cells with the inactive PAF metabolite lyso-PAF did not alter the profile of cell proliferation compared to 10% FBS alone. Thus, lyso-PAF neither inhibited nor stimulated proliferation of the PASMC. However, treatment of the cells with 10 μM of WEB 2170, a PAF receptor antagonist, resulted in significant inhibition of cell proliferation.

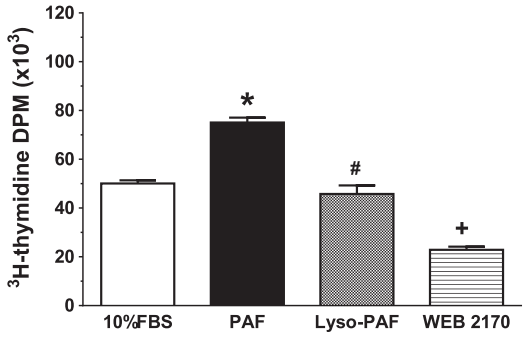

Fig. 2. PAF but not lyso-PAF stimulates proliferation of ovine fetal PASMC. Data are means ± SEM, $n = 5$. Serum deprived cells were studied as described in methods and DNA synthesis was quantified. The statistics are: $^*p < 0.05$, different term 10% FBS control; $\#p < 0.05$, different from PAF effect; and $+p < 0.05$, different from all other treatments.

Fig. 3 shows the effect of PAFR siRNA on PASMC proliferation. Treatment of cells with 10 nM PAF significantly increased cell proliferation compared to 10% FBS control. Treatment of cells with 50 nM of PAFR siRNA significantly decreased cell proliferation by 55% compared to 10 nM PAF and by 18% compared to the effect of 10% FBS. When cells were pulsed for 2 h with the PAFR siRNA and then 10 nM PAF was added, cell proliferation was still statistically less than the effects of 10 nM PAF alone, but no difference compared to 10% FBS alone. Also, co-incubation of the PAFR siRNA and 10 nM PAF did not result in increased cell proliferation compared to 10% FBS control. There was no difference in cell proliferation between the Sham PAFR siRNA and effect of 10% FBS control. However, co-incubation of the Sham siRNA and 10 nM PAF increased cell proliferation by 38% compared to 10% FBS control and 29% compared to Sham siRNA alone. On the whole, co-incubation of the Sham siRNA with 10 nM PAF did not completely reverse the effect of Sham siRNA to the level of effect of 10 nM PAF alone. There was no difference in cell proliferation caused by the Sham PAFR siRNA alone and the 10% FBS control, but the proliferative effect was less than the effect of PAF alone. Thus PAFR siRNA specifically inhibited PAF stimulation of PASMC proliferation.

3.2. Platelet activating factor receptor antagonist WEB 2170 inhibits expression of PAFR, expression and phosphorylation of IkBα, NF-kB p65, and Rb proteins by PASMC

Fig. 4 illustrates the effects of the PAFR antagonist WEB 2170 on expression of non-phosphorylated (Fig. 4a) and phosphorylated IkBα

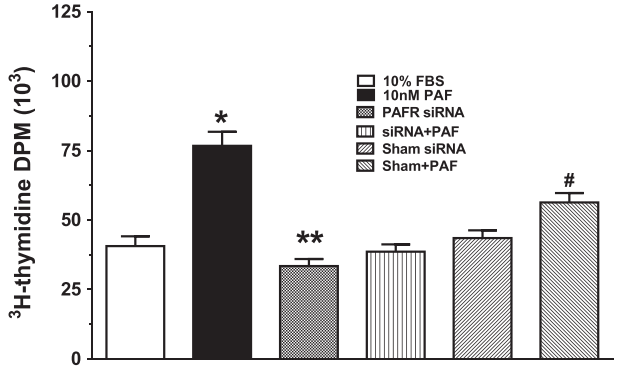

Fig. 3. PAF receptor (PAFR) siRNA attenuated PAF stimulation of PASMC proliferation. Data are means ± SEM, $n = 6$. Sub-confluent cells were transfected with siRNA to PAFR and effect on PAF-stimulation of cell proliferation was examined. PAF treatment augmented cell proliferation whereas PAFR siRNA attenuated PAF stimulation of cell proliferation. The statistics are: $^*p < 0.05$, different from 10% FBS control; $^{**}p < 0.05$, different from PAF treatment and 10% FBS control; $\#p < 0.05$, different from 10% FBS control and Sham siRNA alone.

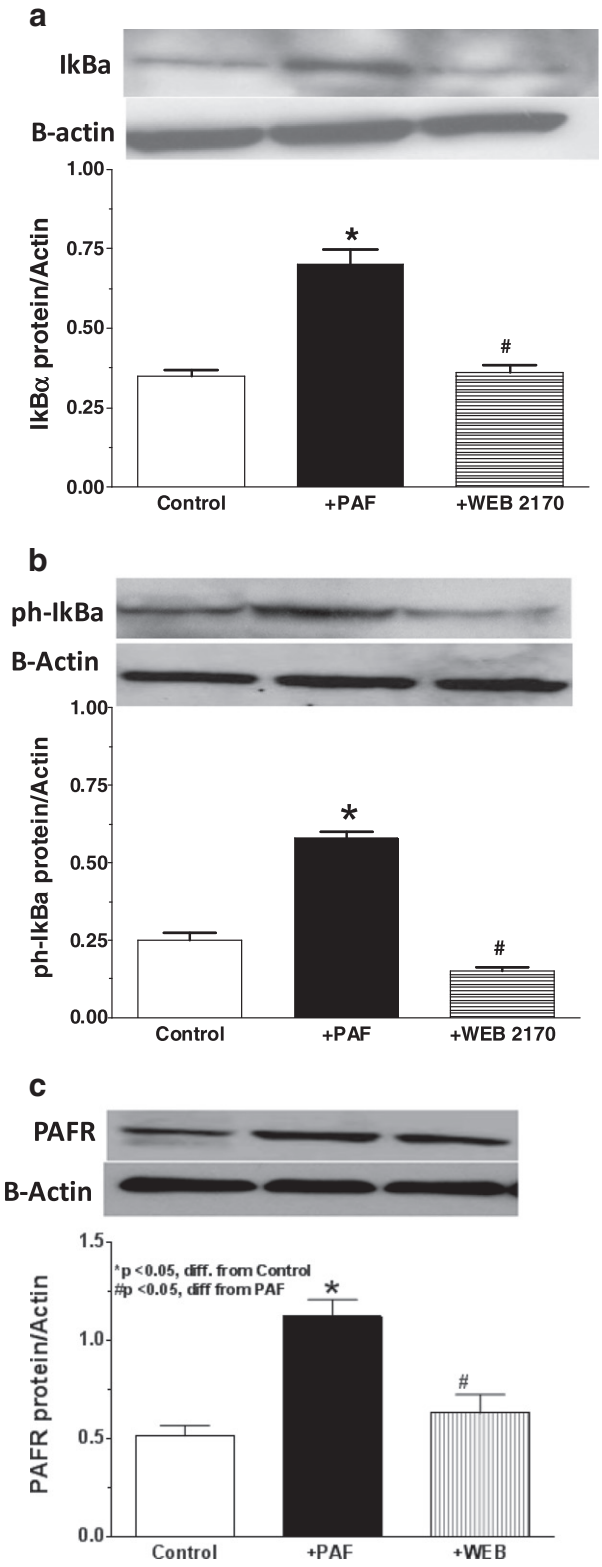

Fig. 4. PAFR antagonist WEB 2170 inhibits expression, panel a, and phosphorylation, Fig. 4b, of IkBα as well as expression of PAFR protein, panel c. Data are means ± SEM, $n = 4$. Sub-confluent cells were incubated for 24 h with 10 nM PAF, or 10 μM WEB 2170 and proteins were isolated and probed for expression of non-phosphorylated IkBα, phophorylated IkBα (ph-IkBα) and PAFR proteins. WEB 2170 inhibited expression and phosphorylation of IkBα and PAFR proteins. The statistics for panels a, b and c are: $^*p < 0.05$, different from 10% FBS control; $\#p < 0.05$, different from PAF treatment and 10% FBS control.

(Fig. 4b), and PAFR (Fig. 4c) proteins. In all three figures, PAF treatment significantly increased PAFR protein expression. However, WEB 2170 treatment significantly attenuated expression of total (non-phosphorylated) IkBα, phosphorylated IkBα (ph-IkBα), and PAFR proteins. The ratios of phosphorylated to non-phosphorylated IkBα were: control, 0.71; +PAF, 0.83; and +WEB 2170 (+WEB), 0.42.

3.3. NF-kB p65 siRNA inhibits PAF stimulation of PASMC proliferation

Fig. 5 shows the effect of NF-kB p65 siRNA on the proliferation of PASMC. Treatment of cells with 10 nM PAF alone caused a 2-fold increase in cell proliferation compared to 10% FBS alone. When cells were treated with 50 nM of NF-kB p65 siRNA alone, cell proliferation was inhibited by 50% compared to the effect of 10% FBS alone and 68% inhibition compared to the effect of 10 nM PAF alone. When the cells were pulsed for 2 h with NF-kB p65 siRNA and then stimulated with 10 nM PAF, the inhibitory effect of NF-kB p65 siRNA was not reversed, proliferation was still 39% of the effect of 10% FBS alone, and 65% inhibition compared to the effect of 10 nM PAF alone, and no significant (<1.0%) change compared to NF-kB p65 siRNA alone. Treatment of cells with Sham NF-kB p65 siRNA alone produced no significant increase (5%) in cell proliferation compared to 10% FBS alone. Co-incubation of Sham NF-kB p65 siRNA and 10 nM PAF produced 33% increase in cell proliferation compared to 10% FBS control, but did not reverse the effect the Sham NF-kB p65 siRNA to the level of PAF treatment alone. Thus the NF-kB p65 siRNA specifically inhibited the ability PAF to induce PASMC proliferation.

3.4. PAFR antagonists inhibit NF-kB p65-DNA binding in PASMC

Fig. 6 shows the effect of PAF on NF-kB-p65 DNA binding. Treatment of cells with 10 nM PAF significantly increased NF-kB p65 DNA binding compared to the effect of 10% FBS alone. NF-kB p65 DNA binding in the presence 100 nM lyso-PAF was not different from the effect of 10% FBS, but significantly less than the effect of 10 nM PAF. On the other hand, when cells were incubated with three different known PAFR antagonists: BN 52021; CV 3988, and WEB 2170, NF-kB p65 DNA binding was significantly inhibited compared to the effect of 10% FBS, 10 nM PAF and 100 nM lyso-PAF. Thus NF-kB p65 DNA binding occurred through a PAFR-linked pathway.

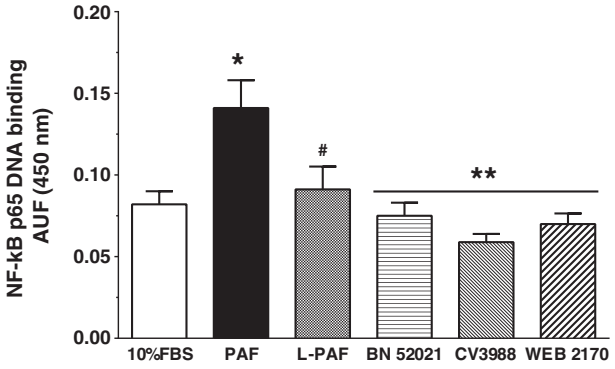

Fig. 6. PAFR antagonists inhibit NF-kB p65 DNA binding to PASMC. Data are means ± SEM, $n = 6$. DNA was isolated from PASMC and subjected to NF-kB p65 DNA binding assay. PAF increased NF-kB p65 DNA binding, lyso-PAF (L-PAF) had no effect on DNA binding compared to effect of 10% FBS control, but three different PAFR antagonists inhibited the DNA binding in a comparable profile. The statistics are: $^{*}p < 0.05$, different from 10% FBS control; $\#p < 0.05$, different from PAF treatment alone; $^{**}p < 0.05$, different from PAF treatment, 10% FBS or lyso-PAF treatment.

3.5. PAF modulates activity of retinoblastoma (Rb) protein in PASMC

Fig. 7 shows the effect of siRNA to Rb protein on PASMC proliferation. Retinoblastoma protein is another nuclear mitogenic protein that acts via PAFR-mediated signaling. Treatment of cells with 10 nM PAF alone caused a 2-fold increase in cell proliferation compared to 10% FBS alone. When cells were treated with 50 nM of the Rb siRNA alone, cell proliferation was significantly inhibited by 35% compared to the effect of 10% FBS alone, 3-fold decrease compared to10 nM PAF alone. When the cells were pulsed for 2 h with Rb siRNA and then stimulated with 10 nM PAF, the inhibitory effect of Rb siRNA was not reversed. Cell proliferation in the presence of 10 nM PAF and 50 nM Rb siRNA increased by 35% compared to the effect of Rb siRNA alone. This was still less than the effect of 10% FBS or10 nM PAF alone. Treatment of cells with the Sham Rb siRNA control alone produced no change in proliferation compared to the effect 10% FBS control. Co-incubation of Sham Rb siRNA and 10 nM PAF increased cell proliferation by 47% compared to sham siRNA alone, but the increase was still below the level of proliferation caused by 10 nM PAF alone. Compared to 10% FBS, co-incubation of 10 nM PAF and the Sham Rb siRNA caused 28% increase in cell proliferation. Thus the Rb siRNA specifically inhibited PAF's ability to induce PASMC proliferation.

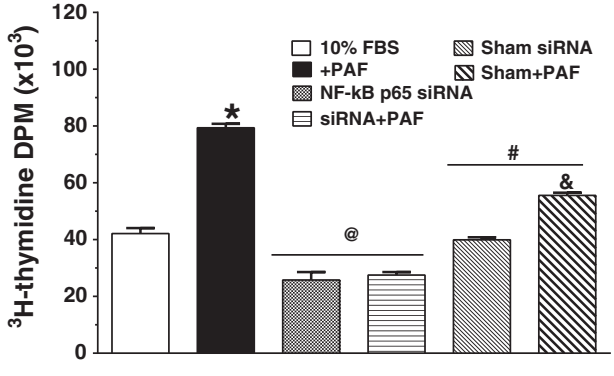

Fig. 5. NF-kB p65 siRNA attenuated PAF stimulation of PASMC proliferation. Data are means ± SEM, $n = 6$. Sub-confluent cells were transfected with siRNA to NF-kB p65 and effect on PAF-stimulation of cell proliferation was examined. PAF treatment significantly augmented cell proliferation whereas NF-kB p65 siRNA attenuated PAF stimulation of cell proliferation. The statistics are: $^{*}p < 0.05$, different from 10% FBS control; $@p < 0.05$, different from 10% FBS and PAF treatment alone; $\&p < 0.05$, different from Sham siRNA alone and 10% FBS control; $\#p < 0.05$, different from 10% FBS control, PAF treatment and NF-kB p65 siRNA treated cells.

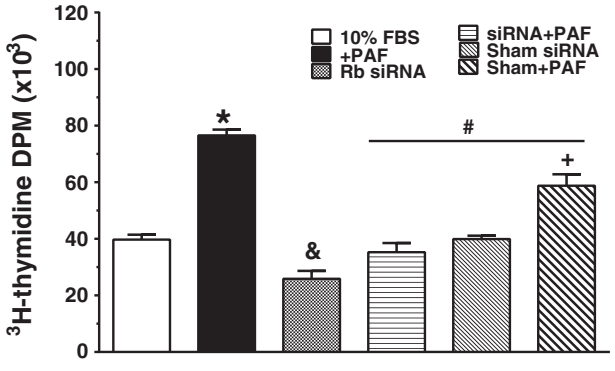

Fig. 7. Retinoblastoma (Rb) siRNA attenuated PAF stimulation of PASMC proliferation. Data are means ± SEM, $n = 6$. Sub-confluent cells were transfected with siRNA to Rb protein and effect on PAF-stimulation of cell proliferation was examined. PAF treatment augmented cell proliferation whereas Rb siRNA attenuated PAF stimulation of cell proliferation. The statistics are: $^{*}p < 0.05$, different from 10% FBS control; $\&p < 0.05$, different from PAF treatment and 10% FBS control; $\#p < 0.05$, different from 10% FBS control, PAF treatment and Rb siRNA treated cells.

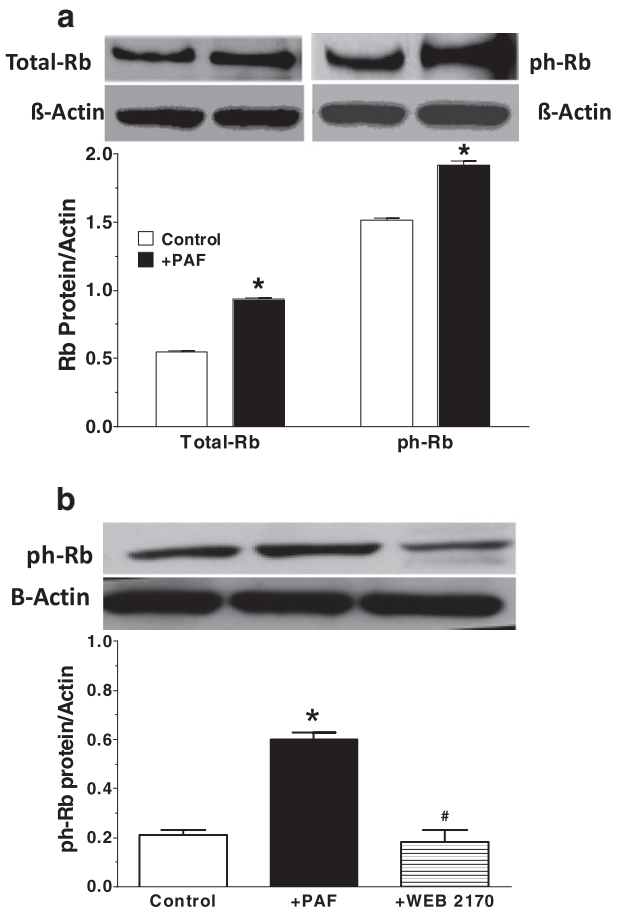

Fig. 8. PAF increases expression of total and phosphorylated Rb protein in PASMC, panel a, and PAFR antagonist WEB 2170 decreased phosphorylation of the Rb protein, panel b. Data are means ± SEM, $n = 4$ different cell preparations. Sub-confluent cells were pulsed for 24 h with either PAF or WEB 2170 and proteins were isolated and probed for total or phosphorylated Rb proteins. In panel a, PAF increased expression and phosphorylation of Rb protein. In panel b, WEB 2170 decreased phosphorylation of Rb protein. The statistics are: $^*p < 0.05$, different from 10% FBS (Control); $\#p < 0.05$, different from PAF or control.

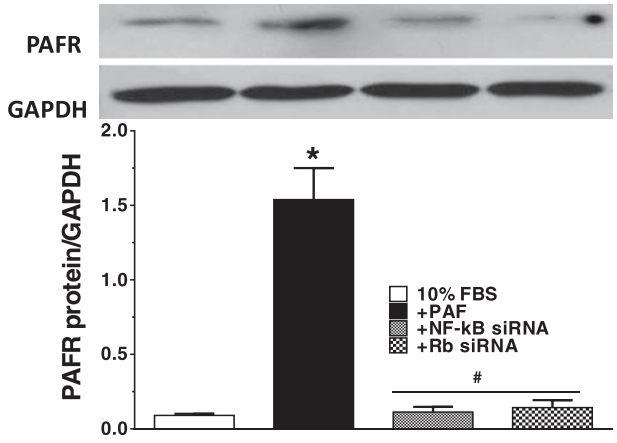

Fig. 9. siRNA to NF-kB p65 and Rb proteins inhibit expression of PAF receptor protein. Data are means ± SEM, $n = 4$. Sub-confluent cells were incubated for 24 h with 10 nM PAF, 50 nM each of siRNA to NF-kB p65 or siRNA to RB protein. Proteins were isolated and probed for expression of PAFR protein expression. NF-kB p65 siRNA and Rb siRNA inhibited expression of PAFR protein. The statistics are: $^*p < 0.05$, different from 10% FBS control; $\#p < 0.05$, different from PAF treatment and 10% FBS control.

Fig. 8 shows the effect of PAF on expression and phosphorylation of Rb protein. Rb protein was expressed under control conditions. Treatment of cells with 10 nM PAF resulted in a 75% increase in total Rb protein expression. The membrane was stripped and probed for phosphorylated Rb protein. Phosphorylation of Rb protein increased both in control conditions and stimulation with 10 nM PAF and then treatment with PAF increased phosphorylation of Rb protein by 27%. Ratio of phosphorylated to total Rb protein expression following 10 nM PAF treatment was: 2.74 for control conditions versus 2.06 following PAF treatment. In Fig. 8b, treatment of cells with 10 nM PAF alone increased ph–Rb protein expression by 65% compared to control conditions. In the presence of WEB 2170, phosphorylation of Rb significantly decreased by 50% compared to the effect of PAF alone, and by 15% compared to the effect of 10% FBS alone.

Fig. 9 shows the effect of 10 nM PAF, 50 nM NF-kB p65 siRNA, and 50 nM Rb siRNA on PAFR protein expression by PASMC. 10 nM PAF significantly stimulated PAFR protein expression compared to 10% FBS control, but NF-kB p65 siRNA and Rb siRNA significantly blunted PAFR protein expression by PASMC.

4. Discussion

During physiological growth and development, proliferation of pulmonary vascular smooth muscle cells plays an important role in normal growth of the vascular system [28,29]. Proliferation of vascular smooth muscle cells is initiated by endogenous and exogenous stimuli, which may involve autocrine or paracrine mechanisms [30]. Induction of cell proliferation by an agent will entail activation of specific signals controlling cell division and cell growth. Platelet activating factor, an endogenous lipid mediator, activates cell growth by autocrine and paracrine mechanisms [30]. We employed pharmacological manipulations in an in vitro setting to examine a possible mechanism by which NF-kB p65 augments PAF-induced pulmonary vascular smooth muscle cell proliferation. The major finding in this study is that siRNAs to PAFR, NF-kB p65 and Rb proteins attenuated PAF stimulation of PASMC proliferation implicating these proteins as endogenous downstream modulators of PAF-induced cell proliferation. Our data show that: a) PAF stimulation of cell proliferation was inhibited by the PAF receptor antagonist, WEB 2170, and by siRNA to PAFR showing that PAF-induced cell proliferation occurred via the PAFR-linked pathway. b) PAF induced nuclear translocation and nuclear DNA binding of NF-kB p65 and NF-kB p65 effect were inhibited by PAFR antagonists showing that NF-kB-p65 effect on cell proliferation is linked to PAFR. c) NF-kB p65 siRNA inhibited cell proliferation and PAFR protein expression, indicating that PAF-induced cell proliferation occurs via NF-kB p65 as the downstream signaling protein. d) siRNA to retinoblastoma protein also inhibited cell proliferation and PAFR protein expression indicating that PAF-induced cell proliferation occurs via Rb protein as a downstream signaling protein linked to NF-kB p65 signaling. Our findings with WEB 2170 together with the effect of CV-3988 and BN 52021 on cell growth and NF-kB p65 DNA binding demonstrate that the effect of PAF on these cells is occurring via PAFR-mediated mechanisms and that the inhibition of PAF stimulation of cell proliferation and PAFR protein expression suggests that the NF-kB p65 siRNA inhibited PAF stimulation of PASMC proliferation by a specific inhibition of PAFR-NF-kB p65 linked signaling.

4.1. NF-kB and PAFR-mediated responses

Physiological and pathological effects of PAF are mediated by its specific G protein coupled receptor reported to be G_q [31]. It has been shown, in previous reports, that PAF induces expression of its own receptors in vivo and in vitro suggesting that PAF activates intracellular molecules that regulate gene expression and cell growth [32–34]. Although hypoxia-inducible factor-1α (HIF-1α) regulates vascular smooth muscle cell proliferation, some other mitogens such as platelet derived growth factor (PDGF), and epidermal growth factor (EGF) also stimulate smooth muscle cell proliferation [11,35]. NF-kB activation can proceed by two different pathways, the canonical pathway whereby

NF-kB activity can be stimulated by upstream effector proteins linked to Ikappa kinase (IKK) proteins, and the non-canonical pathway requiring only Ikkα [36,37]. In a previous report, we showed that NF-kB p65 and cyclin dependent kinases (Cdks) are downstream effectors of PAF-induced pulmonary vascular smooth muscle cell proliferation, with NF-kB p65 presenting an important link between cytosolic and nuclear responses following PAF stimulation of cells [11]. In the present report, we show further evidence to support a role of PAF in nuclear translocation of NF-kB p65 and subsequent activation of nuclear proteins. We show that PAF activates IkBα and its phosphorylation and subsequent induction of nuclear translocation of NF-kB p65 after which NF-kB p65 activates retinoblastoma (Rb) protein and the downstream effectors of PAF-induced PASMC proliferation. The foregoing discussion portends that PAF activation of its membrane receptor induces intracellular signaling pathways that result in PASMC growth and that interruption of this intracellular signal by an endogenous or exogenous molecule should interfere with normal PAF-mediated responses. Our previous findings suggest that Rho kinase may be another protein that acts in the PAFR-mediated signaling pathway to transduce protein activation signals to, perhaps, effect NF-kB p65 translocation into the nucleus [38]. The small GTPase, RhoA, and its effector protein, Rho kinase, are important regulators of vascular reactivity and general pulmonary physiology and pathophysiology [38,39]. In normoxia study involving ROCK effects in pulmonary artery endothelium (PAEC) and PASMC of fetal sheep [39], inhibition of ROCK with Y-27632 led to pulmonary vasodilation even when nitric oxide production was blocked by treatment with N^G-nitro-L-arginine (L-NNA) an enthelium-dependent nitric oxide synthase inhibitor. This suggests that ROCK directly modulates the contraction of fetal PASMC independent of effect of ROCK on PAEC. In another study also involving Rho kinase-ROCK effect on pulmonary vascular smooth muscle cells [38], the ROCK inhibitors Y-27632 and HA-1077 each inhibited PAF stimulation of PASMC proliferation in a similar manner as quantified by either direct assessment of DNA synthesis or by immunocytochemistry. RhoA regulates various cell functions including cell growth and gene expression [40]. Inhibition of PAF-stimulation by ROCK in PASMC suggests that in an in vivo condition in fetal lungs the needed pulmonary vascular hyperplasia may not occur, whereas in newborn lungs this will be a desirable condition as it will mitigate cell growth thereby preventing hyperplasia which will ultimately result in PPHN.

We have shown that in ovine fetus, PAF acts through its receptors in the lung to maintain a high pulmonary vasomotor tone in ovine fetal hypoxic lung environment in vivo and inhibition of the PAFR-mediated effect resulted in decreased pulmonary artery pressure with concomitant increase in pulmonary blood flow [10]. We have also shown that, in vitro, PAF acts through its receptors to increase ovine fetal pulmonary vascular smooth muscle cell proliferation and calcium release [11,41]. Thus a high PAFR-mediated activity is necessary to maintain a high pulmonary vasomotor tone in fetal lungs. This will mean that inhibition of PAFR-mediated effects in ovine fetal smooth muscle in vivo, even in a nonspecific mechanism, presents a detrimental condition for ovine fetal lung function and development [10,42,43]. Our findings that NF-kB p65 and PAF inhibitors decrease PAFR protein expression and PAFR-mediated responses, suggest the existence of a PAF–PAFR–NF-kB p65 interaction in fetal pulmonary circulation. A disruption of this congenial interaction will result in adverse fetal pulmonary circulatory conditions. Abnormal regulation of the functions of pulmonary vascular smooth muscle and endothelium, for example inability to down-regulate production of vasoconstrictors by the vascular smooth muscle and endothelial cells and up-regulate endothelium-derived vasodilators, in the perinatal period is implicated in the pathogenesis of persistent pulmonary hypertension of the newborn [PPHN]. Therefore down-regulation of PAFR-mediated responses postnatally, by inhibition of NF-kB p65 activity, may be one mechanism to prevent the pulmonary vascular abnormalities of PPHN. Persistent pulmonary hypertension of the newborn is a pathological condition with different etiologies [5,23,24,44,45]. Neonates with PPHN have high PAF levels, showing that persistence of high PAF levels postnatally leads to abnormal perinatal pulmonary adaptation. We speculate that in vivo, in the hypoxic environment of fetal lungs where PAF level is high, PAF will act via its receptor in conjunction with the activities of Rho kinase, to induce proliferation of smooth muscle cells, an important role in the maintenance and remodeling of the fetal pulmonary vascular system during the 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 resulting in incidence of PPHN. We further speculate that RNAi to PAFR and its downstream signaling proteins may offer a means to prevent or manage pulmonary vascular abnormality such as pulmonary vascular hyperplasia in some lung diseases and PPHN where PAFR-mediator signaling is implicated. This can be achieved, in vivo, by administering PAFR siRNA, which should decrease or abolish PAFR-linked signaling and thereby decrease the incidence of PPHN.

Acknowledgments

This work was supported in part by grant 513292 by the Los Angeles Biomedical Research Institute, Torrance, CA.

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