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

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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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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.

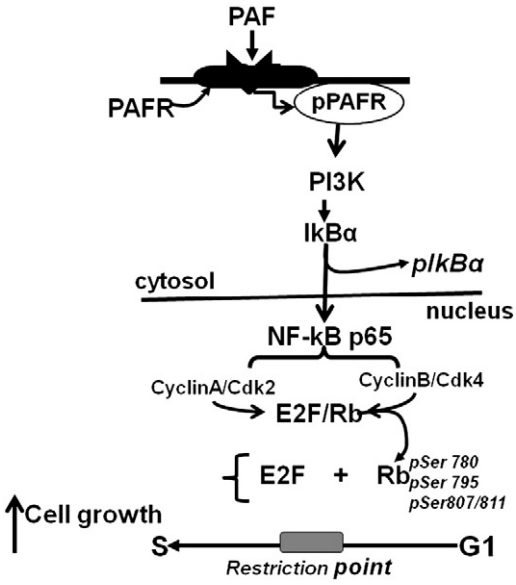

Fig. 1. Scheme of study hypothesis.

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