RESEARCH ARTICLE

Physiological and Biochemical Consequences of Exposure of Neonatal Rats to Chronic Hypoxia

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Abstract

We studied effect of chronic hypoxia (CH) on expression of platelet activating factor receptor (PAFR) by neonatal rats (pups). We hypothesized that PAFR antagonist will prevent pulmonary hypertension (PH) in pups exposed to CH. Pups were placed in an air-tight chamber ventilated with 13% oxygen, hypoxia (Hpx) or room air normoxia (Nmx) from 1d to 22d of age. Three groups of pups were studied (each group, n=10-14 pups): Group1, pups in Nmx; Group2, pups in Hpx given 5mg/kg PAFR receptor antagonist, WEB 2170, IP, every other day for 22d, (Hpx+WEB); Group3, pups in Hpx control. Hemotocrit, RV/LV+S, PAF binding, PAF synthesis, and PAFR expression were determined. Hyx control group had 2-fold higher RV/LV+S than Nmx group and PAFR antagonist decreased RV/LV+S to the Nmx control value. Lungs of pups in Hpx expressed more PAFR protein than Hpx+WEB and Nmx groups. Additionally, Hpx increased PAF synthesis and PAFR binding whereas WEB treatment decreased PAFR binding, but produced no difference in PAF synthesis compared to Nmx group. Hpx increased NF-kB p65 and TLR4 expression. WEB treatment abrogated expression or NF-kB p65 and TLR4 proteins. Our findings show that chronic hypoxia induces expression of PAFR, NF-kB p65 and TLR4 by pups' lungs and suggest that increased PAFR expression may be responsible for the right ventricular hypertrophy and PH. Thus a PAFR antagonist may offer a therapeutic intervention for CH-induced PH in human neonates.

Key words: chronic hypoxia, hypertrophy, PAF receptor, NF-kB, TLR4

Introduction

The pulmonary vasculature responds to a variety of endogenous vasoactive compounds, such that an imbalance of endogenous vasoconstrictors and vasodilators in the pulmonary vasculature of the newborn, may predispose the pulmonary circulation to vasoconstriction and pulmonary hypertension.[1-4] Neonatal pulmonary hypertension is a disease with high morbidity and mortality[5,6] and it induces pulmonary vascular remodeling and right ventricular hypertrophy.[7] It has been known for a long time that exposure to chronic hypoxic condition induces pulmonary vascular hyperplasia, remodeling and subsequently pulmonary hypertension.[8] In adult rats, platelet activating factor receptor (PAFR) antagonist prevented chronic hypoxia-induced pulmonary hypertension,[9-11] implicating endogenously synthesized PAF in this pulmonary vascular abnormality. Platelet activating factor, 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (PAF), is a phospholipid with diverse physiological actions.[8] High plasma PAF levels have been reported in newborns with persistent pulmonary hypertension of the newborn (PPHN), with a fall in PAF levels as they improved clinically.[9] PAF has also been implicated in chronic hypoxia-induced pulmonary hypertension (PH) in adult rats.[10,11] In those studies, adult rats placed in chronic hypoxia developed PH and right ventricular hypertrophy.[10] Furthermore, PAF receptor (PAFR) antagonists blunted hypoxia-induced PH and pulmonary vascular remodeling in adult rats,[10] indicating a PAFR-mediated mechanism.

In a previous report, we showed that PAF level in the fetal lamb pulmonary vasculature is high and the level falls significantly after birth and by using specific PAFR antagonists infused into fetal lambs *in vivo*, it was demonstrated that PAF acting via its specific receptors contributes significantly to maintenance of high vasomotor tone in the pulmonary circulation *in utero*.[12] Furthermore, it was shown that chronic *in utero* hypoxia up-regulates PAF binding and PAF receptor expression in pulmonary vascular smooth muscle cells of fetal lambs.[13] These findings support a unique role for PAF as an important endogenous mediator of high pulmonary vasomotor tone in the fetus, and suggest that a down regulation of PAF and PAF receptor (PAFR) expression will contribute to amelioration of PPHN. Nuclear factor κappa Beta (NF-kB) is a family of transcription factors that regulate a variety of cell functions.[14] The family includes RELA (NF-kB p65) which is the most studied with respect to its involvement in inflammation and cell growth. We and others have demonstrated that NF-kB p65 is an important mediator of pulmonary arterial smooth muscle cell growth and is impacted by hypoxic conditions.[15-18] Therefore, we investigated the effect of chronic hypoxia exposure on NF-kB p65 protein expression by the neonatal rat lungs. Also, it has been shown that expression of NF-kB p65 and TLR4 proteins may exhibit a crosstalk signaling in pulmonary arterial smooth muscle cells in hypoxia. Therefore, we also determined effect of chronic hypoxia on TLR4 protein expression by lungs of these pups.[19,20] We hypothesized that PAFR antagonist, such as WEB 2170 will prevent the onset of pulmonary hypertension (PH) in neonatal rats exposed to CH by blocking biosynthesis of mediators and proteins in the PAF bioactivity pathway. Therefore, we tested this hypothesis that exposure of neonatal rat pups to chronic hypoxia up-regulates PAF and PAFR protein in the pulmonary vasculature of the rat pups.

Materials

The experimental protocol was approved by the Institutional Animal Care and use Committee of the Lundquist Institute. Pregnant Sprague Dawley rats were purchased from Charles River (San Diego, CA). Authentic PAF (C_{16}-PAF) and lyso-C_{16}-PAF standards were purchased from Biomol laboratory (Plymouth Meeting, PA). Radiolabeled PAF standards and substrates; hexadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine, 1-O-[acetyl-^{3}H-(N)]-, (^{3}H-acetyl-C_{16}-PAF), 21.5 Ci/mmol (370 GBq/mmol) were purchased from Perkin Elmer Life Sciences (Boston, MA). Antibody to Toll Like Receptor 4 (TLR4) was purchased from Santa Cruz Biotechnology (Dallas, TX).

Antibody to NF-KB p65 was purchased from Cell Signaling Technology (Danvers, MA), while PAF receptor antibody was purchased from Cayman Chemical Company (Ann Arbor, MI). The PAFR antagonist WEB 2170 was kindly donated by Boehringer Ingelheim (Ridgefield, CT). Ecolite(+) liquid scintillation cocktail was purchased from MP Biochemicals (Irvine, CA).

Methods

Pregnant Sprague Dawley rats at e18 were allowed to acclimatize to the vivarium environment and deliver in room air at the C.W. Steers Biological Resource Center of the Lundquist Institute. The cages were examined daily for presence of pups early morning and late evening. As soon as pups were present, they were divided into study groups and placed with their mom in the necessary study environment: cages in room air and cages in hypoxia chamber.

Study groups

Three groups of pups were studied.

Group 1: Pups in normoxia, identified as normoxia controls (Nmx control). These pups served as the controls for pups studied in hypoxia.

Group 2: Pups in hypoxia given 5mg/kg WEB 2170, intraperitoneal (IP), every other day and identified as hypoxia +WEB (Hpx+WEB).

Group 3: Pups in hypoxia without treatment and identified as hypoxia controls (Hpx control).

Treatment protocol

For hypoxia studies, pups and mothers in a nursing cage were placed in an air-tight transparent hypoxia environment and ventilated with 13% O_2, balance nitrogen for 22 days from day1 of birth (1d) to day22 of age (22d) which was also the day22 of treatment. Various concentrations of oxygen (10%-15%) were tested for satisfactory outcome as regards stable hypoxic chamber environment, mortality of pups, and stable weight gain, from which 13% oxygen was determined as the oxygen concentration that caused the least mortality of the pups with stable hypoxic environment of the ventilation chamber. Food and water were provided ad libitum and the pups were allowed unrestricted nurturing by the mothers. One group of pups, the Hpx+WEB group received the PAF receptor antagonist, WEB 2170 (WEB), 5mg/kg, intraperitoneal (i.p), every other day for 3wks. The hypoxia control group received equivalent volume of normal saline every other day. A bowl containing soda lime was placed in the hypoxia chamber to absorb carbon dioxide during the ventilation period. Oxygen saturation in the hypoxia chamber was between 12% and 13%, and was continuously monitored with TED 60T percent oxygen sensor, Teledyne Analytical Instruments (City of Industry, CA).

For the normoxia control studies, pups with their mothers in the cage were placed in room air for 22 days and treated with equivalent volume of saline (sham). The pups of the three groups were allowed unrestricted nurturing by their mothers.

The pups were weighed before being placed in the hypoxia chamber or in room air and then they were weighed every week throughout the duration of the study. The pups were monitored daily, the cages were cleaned every morning and fresh supply of water and food were provided daily.

Study protocol

On day 22 of exposure to hypoxia or room air, pups were weighed and euthanized by i.p. injection with 100mg/kg pentobarbital and then blood was drawn from each pup by cardiac puncture for determination of hematocrit. Lungs and heart were excised with the heart and then the heart alone was weighed. The heart was cut open to determine weight of right ventricle (RV) and left ventricle plus septum (LV+S), and then RV/LV+S ratio was calculated. The lungs were used for further studies.

Physiological measurement: Pulmonary artery (PA) preparation and vessel tension studies. Vessel tension studies were done essentially as we previously reported.[21] Briefly, immediately after euthanasia, lungs were removed and third- to fifth-generation PA are isolated, placed on ice-cold modified Krebs-

Ringer bicarbonate buffer (Krebs buffer), pH 7.4, and dissected free of parenchyma. Vessels were cut into 4-5mm rings and stored in the ice-cold Krebs buffer and used for experiments. Outside diameters of PA of controls and experimental groups were determined. Vessel rings were suspended in organ chambers filled with 10 ml of the modified Krebs buffer maintained at 37 ± 0.5°C and aerated with 95% O_2-5% CO_2 (pH 7.4). At the beginning of the experiment, each vessel ring was stretched to its optimal resting tension, achieved by step-wise stretching, in 0.1-g increments, until the contractile response to 100 mM KCl plateaus. Then vessels are allowed to equilibrate for one hour. Effects of PAF on the pre-constricted vessels are determined on PA pre-constricted with endothelin-1 (ET-1, $3x01^{-9}$) to a similar tension. In all experiments, indomethacin (10^{-5} M) is added in the organ chamber to exclude possible interference of vasoactive prostanoids.

Measurement of PAF binding, PAFR expression, expression of nuclear factor-kappa beta (NF-kB) p65 and Toll Like Receptor 4 (TLR) 4 proteins.

Lungs from each pup were perfused to rid the vasculature of blood cells until the lungs appeared bleached. Washed lungs were then homogenized as we previously reported[22] and the lung protein was used to measure expression of PAFR, NF-kB p65 and TLR4 by Western blotting.

SDS-PAGE electrophoresis: Protein expression was quantified by a modified coomassie blue technique[22,23] before Western blotting. SDS-PAGE electrophoresis was done on 4-12% Tris-glycine gradient gels (Lonza, Rockland, ME), as we previously described.[22] Membranes were probed for PAFR, NF-kB p65, and TLR4. Antibodies to PAFR were purchase from Cayman Chemical (Ann Arbor, MI) anti-rabbit Ig HRP-linked secondary antibody was purchased from Bioss (Stoughton, MA). Signals were captured with Amersham ECL Western Blot detection kit on X-ray film. The membranes were stripped and re-probed for β-actin which was used as the internal standard. Each protein was quantified against expression of the β-actin standard or GAPDH internal standard. Bands corresponding to PAFR, NF-kB p65, and TLR4 were scanned with an UnscanIT program to quantify blot density. Protein expression is presented as the ratio of the specific protein to β-actin standard.

Assay of PAF receptor binding

PAFR binding was measured in the membrane fraction of lung homogenate. Briefly, lung homogenate was spun in a refrigerated centrifuge at 300xg for 5 min. The resulting supernatant was centrifuged at 9,000g for 20 min in refrigerated centrifuge. The 9,000g pellet was re-suspended in 10 mM Tris buffer with 1 mM PMSF and 25mM sucrose and spun for 1 hr at 100,000g at 4^{O}C to pellet the membrane protein, which was then re-suspended in the sucrose buffer and used for radioligand binding assay of PAFR as we previously reported.[22]

Assay of PAF synthesis by pulmonary arteries

Pulmonary arteries isolated from the lungs of the neonatal rats were pooled and used to study PAF synthesis by measuring the activity of lyso-PAF:acetyl-S-CoA acetyltransferase in the membrane proteins as we previously reported. We studied PAF synthesis by membrane protein of the isolated pulmonary arteries (PA) only, because we wished to correlate PAF level to its site of action in the vessels, *in vivo*. Briefly, 250μM aliquot of lyso-PAF and 500μM [^{3}H]-acetyl-S-CoA (21.5 Ci/mmol) were placed in a polypropylene tube and warmed to 37^{O}C in a shaker bath. Reaction was initiated by adding 100μg of membrane protein of each treatment for a total volume of 1 ml of 30 mM Tris buffer, pH 7.4, containing 10 μM $CaCl_2$, 1 mM dithiothreitol, and 0.025% BSA and incubating the mixture at 37^{O}C for 15 min. After incubation, [^{3}H]-acetyl-PAF synthesized was subjected to lipid extraction as we previously reported.[18]

Data Analysis

All numerical data: hematocrit, RV/LV+S, PAFR binding and PAF synthesis, etc, were quantified and presented as means ± SEM and were analyzed for statistical significance with a two-tailed student t-test. In studies with radioactive compounds, background

radioactivity was subtracted before quantifying the data. Dose response effect of PAF ($1x10^{-9}$ to $1x10^{-6}$) on PA rings from Nmx control and Hpx control are presented as change in optimal resting tension, means ± SEM, and subjected to ANOVA statistics at $p < 0.05$ level of statistical significance. Expression of PAFR, NF-kB p65 and TLR4 proteins was normalized to expression of actin standard and analyzed for statistical significance, also at $p < 0.05$ level of statistical significance.

Results and Discussion

Physiological implications of chronic hypoxia exposure of pups: Chronic hypoxia exposure resulted in reduced body weight compared to hypoxia control (Hpx control) and Hpx+WEB pups compared to the Nmx control group, but there was no significant difference in body weight between the Hpx control group and the Hpx+WEB group. The values were body weight gm: Nmx control, 54.39 ± 1.37; Hpx control, 36.97 ± 1.26; Hpx+WEB, 34.84 ± 1.26. After establishing 13% oxygen as the congenial hypoxia oxygen environment for the study, there was no mortality of the pups during the duration of the study. Figure 1a shows the hematocrit values of the three groups of pups. In Nmx control, the hematocrit was 30.30%, which increased to 42.20% in the Hpx group, more than 30% greater than that of the Nmx control pups. WEB 2170 treatment (Hpx+WEB) brought the hematocrit down to the level of the Nmx controls. The index of hypertrophy, RV/LV+S, for the three groups of rat pups is shown in Figure 1b. The RV/LV+S ratio for the chronic hypoxia control (Hpx control) pups was >2-fold more than for the Nmx control pups. Treatment with WEB 2170 (Hpx+WEB) brought the hypertrophy index of the chronic hypoxia (Hpx control) group down to the level of the Nmx controls. The higher hematocrit in hypoxic environment of the pups indicates existence of polycythemia.[24,25] Increased hematocrit will suggest an increased platelet count, which may result in exaggerated PAF production under this condition. The pups showed significant left ventricular hypertrophy. It is not certain whether this is an adaptive condition for the pup hearts to accommodate the increased hematocrit. Regardless of the underlying reason, the hypertrophic condition indicates a serious physiological maladaptation of neonatal cardiopulmonary circulation in a hypoxic environment. Of note is the fact that the administration of the PAFR antagonist WEB 2170 obliterated the high hematocrit and abolished the hypertrophic conditions in the treated pups in hypoxia. These findings suggest that a PAF receptor antagonist such as WEB 2170 will offer a congenial therapeutic intervention for chronic hypoxia-induced cardiac cum pulmonary hypertension, more especially in the neonate. We then investigated the response of isolated and pre-constricted pulmonary arteries to exogenous PAF. Figure 1c shows the effect of chronic hypoxia on isolated vessel tension studies of pulmonary arteries (PA) of pups subjected to chronic hypoxia without WEB 2170 treatment (PA HPX control) and compared to activity of pups subjected to normoxia (PA Nmx control). Response of the PA from chronic hypoxia pups was concentration dependent. Compared to PA from normoxia group, exposure to chronic hypoxia increased the optimal resting tension of the chronic hypoxia group at all the concentrations tested. The response of Nmx control vessels to PAF treatment was not significantly different to baseline until higher concentrations of PAF. Etiologies of pulmonary hypertension of the neonate are varied and they are not clearly understood, but the condition is marked by a high morbidity and mortality of the presenting newborns.[26-28] Chronic *in utero* exposure to high altitude environment[13,16] and epigenetic factors[29] are some natural causative factors of neonatal pulmonary hypertension. Among other things, the data presented here show that chronic hypoxia exposure alone is sufficient to induce neonatal pulmonary hypertension in support of existing information and further show, for the first time, that chronic neonatal hypoxia exposure contributes to increase in pulmonary vascular tone of newborns. This increased tone will result in increased pulmonary vascular resistance in vivo

and will lead to the condition of pulmonary hypertension of the newborn.

Figure 1: Physiological data from neonatal rats exposed to chronic hypoxia

Figure 1a: Hematocrit measurement

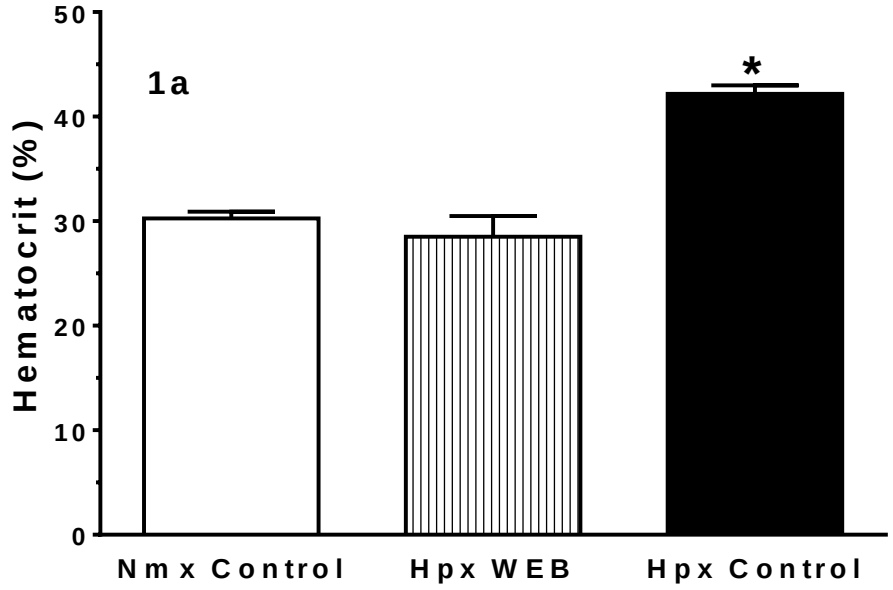

Figure 1b: Index of hypertrophy (RV/LV+S)

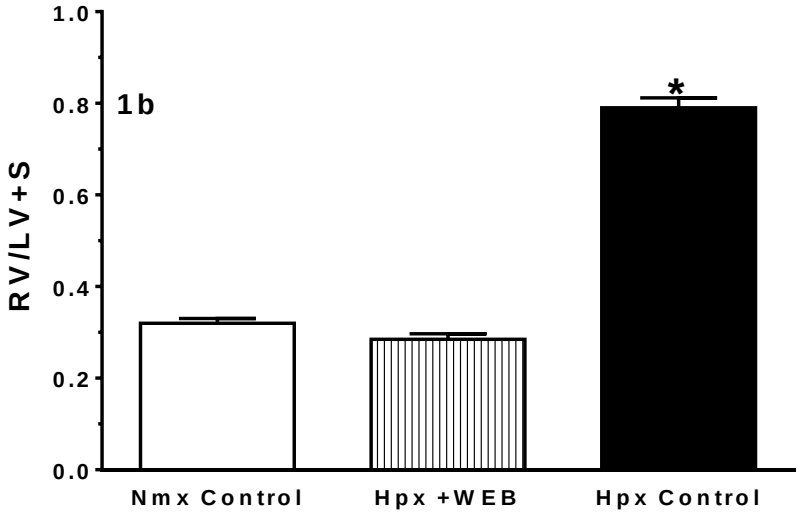

Figure 1c: Effect of chronic hypoxia exposure on optimal resting tension study

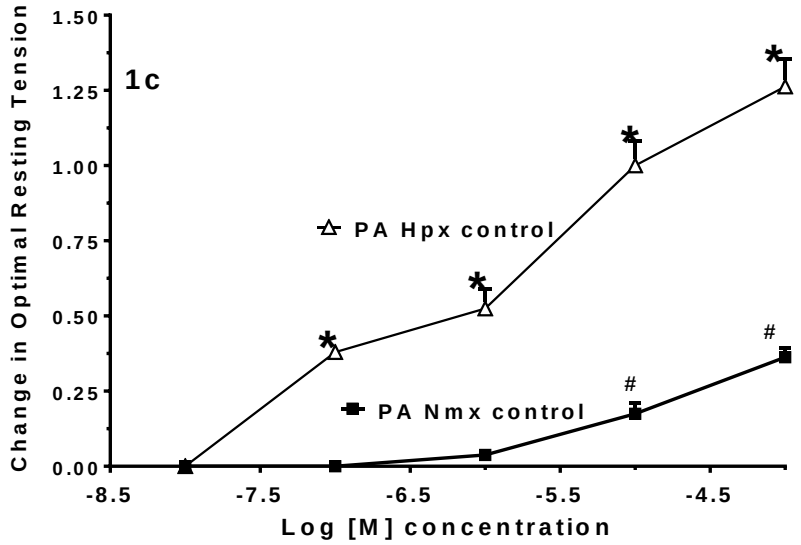

Figure 1 shows the physiological data resulting from the neonatal rats exposed to chronic hypoxia for 22 days. Data are means ± SEM, n =10. On day 22 of hypoxia or room air exposure, pups weights were determined, pups were euthanized and blood was drawn from each pup by cardiac puncture for determination of hematocrit. Figure 1a shows the hematocrit (%) values of the three groups of pups. In normoxia control (Nmx control), the hematocrit was 30.30 ± 0.66, hypoxia control (Hpx control), 42.25 ±0.81 and Hpx+WEB, 28.50 ± 1.99. Hematocrit of Hpx control pups was significantly higher than for Nmx control or Hpx+WEB pups. Figure 1b shows index of hypertrophy (RV/LV+S) determined from each group of pups. The values were, Nmx control, 0.32 ± 0.032; Hpx+WEB, 0.29 ± 0.36; Hpx control, 0.79 ± 0.058. The hypertrophy index of the chronic hypoxia (Hpx control) pups was significantly greater that the Nmx control or Hpx+WEB pups. Figure 1c shows the change in optimal resting tension for pulmonary artery vessel segments of Nmx control and Hpx control pups. The vessels from the Hpx control pups were significantly more constricted to PAF effect than vessels from the Nmx control pups. *p <0.05, different from Nmx control or Hpx+WEB; #p <0.05, different from preceding concentration for Nmx control

Biochemical implications of pulmonary exposure to chronic hypoxia

PAF binding assays: Effect of chronic hypoxia exposure on PAF binding to its receptors in lung membrane proteins of the three groups of neonatal pups is shown in Figure 2 and expressed in femtomole/μg protein (fmol/μg protein), means ± SEM. Binding to lung membranes of the chronic hypoxia (Hpx control) control pups, Figure 2a, was 9.51 ± 2.00, which is >2-fold more than binding to membranes of the Nmx control group. Treatment of the pups with WEB 2170 during hypoxia exposure (Hpx+WEB) greatly inhibited PAF binding to the neonatal pups lung membranes 0.31 ± 0.051 which was >10-fold less than binding to lung membranes of the Nmx controls, and >30-fold less than binding to lung membranes of the Hpx control group. Of note is the fact that these lung membranes of the Hpx+Web group was not pre-treated with exogenous WEB 2170 during receptor binding assay before PAF binding assay was done. We then tested PAF binding to its receptors in membranes of pulmonary arteries isolated from the three groups of pups. Vessel membrane proteins were prepared from pulmonary arteries (PA) isolated from the three groups of pups, Figure 2b. PAF binding to PA membranes of Hpx controls was 2.00 ± 0.40, which was greater than binding to PA membranes of the Nmx control group, 0.80 ± 0.088. As with the

lung membranes proteins, WEB 2170 treatment during hypoxia exposure, Hpx+WEB, decreased PAF binding to PA membranes. The value was 0.078 ± 0.014. Thus, whether in lung membranes or PA membranes, chronic hypoxia exposure of pups with WEB 2170 treatment dampened PAF receptor binding.

PAF synthesis: The anabolism of PAF by membrane proteins from the 3 groups of pups was determined by measuring the activity of the PAF synthesizing enzyme, lyso-PAF:acetyl-S-CoA acetyltransferase in membrane proteins as we previously reported.[23] PAF synthesis by membranes proteins of the PA of the three groups is shown in Figure 2c, and presented as pmol/µg protein. In PA membranes of Hpx control, PAF synthesis was 9.51 ± 1.75, which was over 60% greater than synthesis by Nmx control membranes. PAF synthesis by PA membranes of Hpx+WEB pups was 4.86 ± 0.64 which was significantly less than synthesis by Hpx control, but was not significantly different from synthesis by Nmx control, $p>0.05$. Thus, WEB treatment during hypoxia exposure inhibited adequate lyso-PAF production from the requisite endogenous phospholipid. At present we cannot conclude on the relevant point in endogenous phospholipid biosynthesis controlling the level of lyso-PAF in the Hpx+WEB PA vessels. However, it has been shown that oxygen tension influences phospholipid catabolism.[30,31] This will result in a lower concentration of lyso-PAF that will be available for PAF synthesis by the remodeling pathway, which we have examined in this report. We can infer from the pieces of information from Figures 2a, 2b, and 2c that *in vivo* PAF, as an endogenous molecule, is capable of stimulating its own receptor protein expression, thereby making PAF receptor protein available for PAF binding. Such a condition will sustain PAF effects in a hypoxic condition and thus endow the neonate, in this case the pups, with the condition of pulmonary hypertension. Interestingly, profile of PAF binding to membranes of PA from Hpx+WEB corroborates the results of the tension studies with isolated vessels, Figure 1c. This is a new information in the quest to understand the pathology of pulmonary hypertension of the newborn.

2: Biochemical data from neonatal rats exposed to chronic hypoxia

Figure 2a: PAF binding to its receptors in lung membranes of neonatal rats exposed to chronic hypoxia

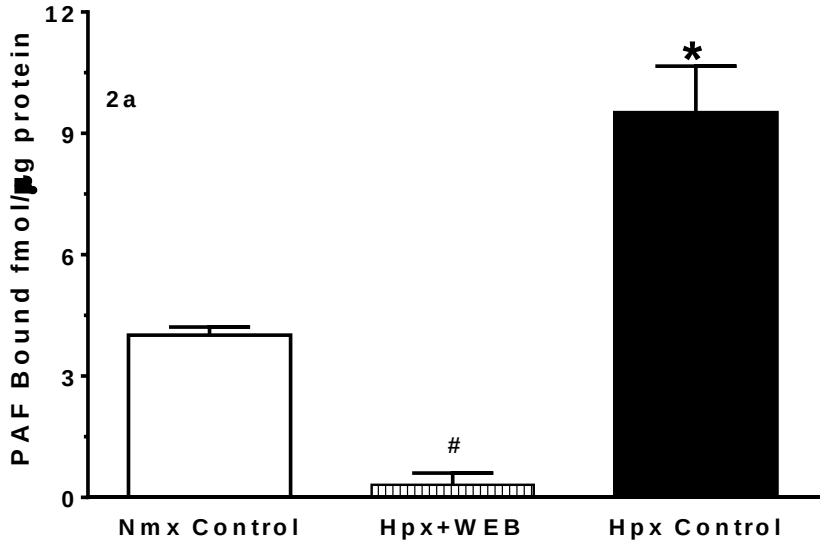

Figure 2b: PAF binding to its receptors in PA vessel membranes of neonatal rats exposed to chronic hypoxia

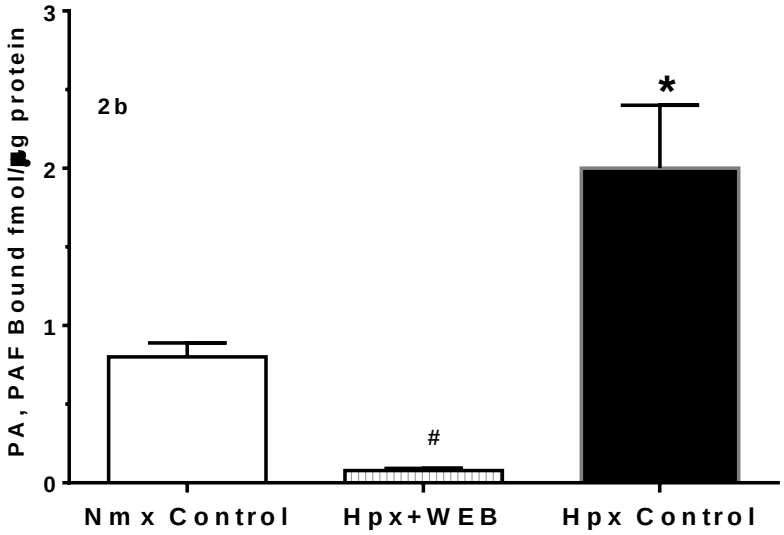

Figure 2c: PAF synthesis by PA vessel membranes of neonatal rats exposed to chronic hypoxia

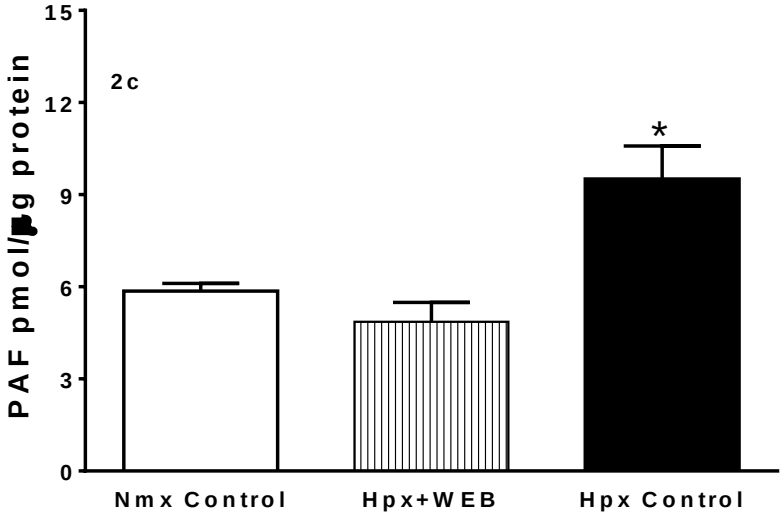

Figure 2 shows the PAFR binding assays and the PAFR protein expression assays. Data are means ± SEM, fmol/µg protein, n =14. Membrane proteins were prepared from freshly isolated lungs from the three groups of pups. Figure 2a shows that PAF binding to lung membrane proteins of Hpx control pups was 9.51 ± 2.00. The value for Nmx control was 4.01 ± 0.35, while the value for Hpx+WEB was 0.31 ± 0.057. WEB 2170 treatment in hypoxia significantly inhibited PAFR binding. Figure 2b shows values of PAFR binding to membrane proteins of isolated pulmonary artery (PA) from the three groups of pups. Binding to Hpx control PA membranes was 2.00 ± 0.40, which was greater than for Nmx control PA, 0.80 ± 0.09 or Hpx+WEB, which was 0.078 ± 0.014. For clearer visual appreciation, the plotting scale used for the lung membrane data is different from that for PA membrane. PAF synthesis pmol/µg protein by PA membranes of the three group of pups is shown in Figure 2c. Synthesis by Hpx control pups was 9.51 ± 1.075, which was significantly greater than 5.86 ± .26 synthesized by PA of Nmx control pups. Value for PA from Hpx+WEB pups was 4.86 ± 0.63. *p <0.05, different from Nmx control or Hpx+WEB; #< 0.05, different from Nmx control.

Protein expression studies: Figure 3 shows effect of chronic hypoxia exposure on PAF receptor protein expression by lung membranes of the 3 groups of pups. Protein expression was quantified and expressed as ratio of the corresponding actin expression (protein/Actin or protein/GAPDH, means ± SEM). PAF receptor (PAFR) protein expression by lung membranes, Figure 3a, shows that the Hpx control group expressed significantly more PAFR protein than the Nmx control; 1.26 ± 0.054 by Hpx control group versus 0.81 ± 0.060 by the Nmx control group. Chronic exposure of the pups to hypoxia with WEB 2170 treatment significantly decreased PAFR protein expression by the Hpx+WEB group, 0.51 ± 0.032, compared to either the Hpx control or the Nmx control as shown above. Figure 3b, shows the PAFR protein expression by proteins from isolated PA vessels from the three groups of neonatal pups also measured by Western blotting and presented as for the lung membrane proteins. PAFR protein expression by the Hpx control pups was significantly greater than expression by the Nmx control pups. Treatment with WEB 2170 during hypoxia exposure significantly decreased receptor protein expression by the Hpx+WEB compared to Hpx control or Nmx control groups. This indicates that the PAF receptor antagonist WEB 2170 not only prevents PAF receptor binding under *in vitro* receptor binding assays,[32] but also inhibits nascent PAF receptor protein expression in the chronic hypoxia lung environment of the pups. This is a novel finding as it demonstrates existence of a complex pathway whereby a chronic hypoxic environment can impact the normal physiology and biochemistry of neonatal lung function, perhaps including adult lung function as previous reported.[11]

Figure 3: PAF receptor protein expression by lung and vessel membranes of neonatal rats exposed to chronic hypoxia

Figure 3a: Lung membrane proteins

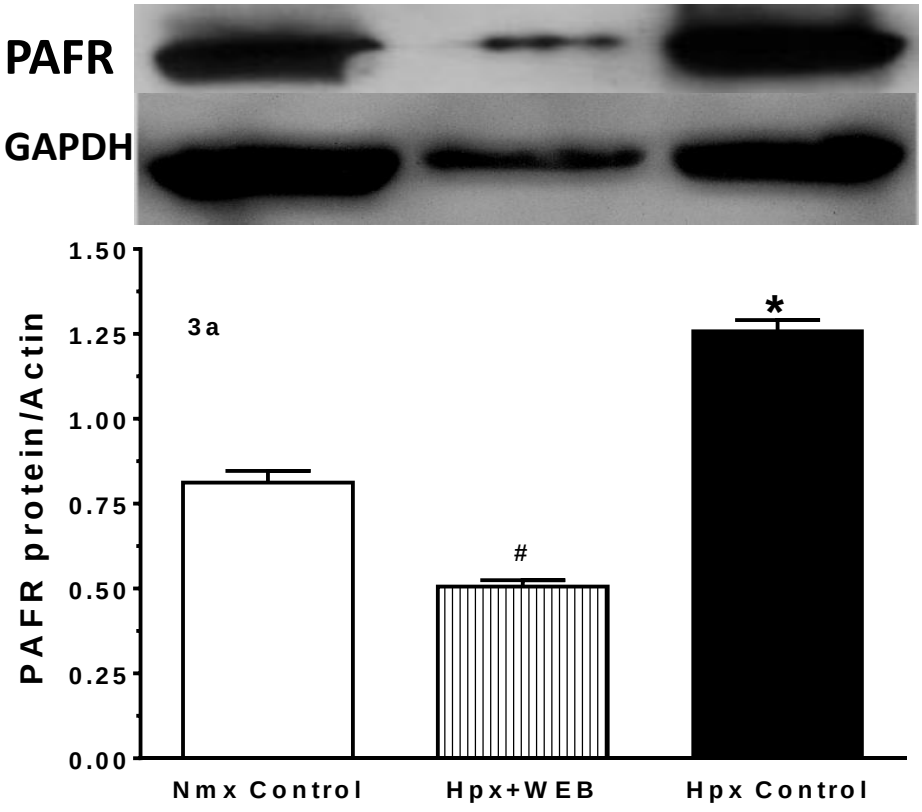

Figure 3b: PAF receptor protein expression by PA vessels of neonatal rat exposed to chronic hypoxia.

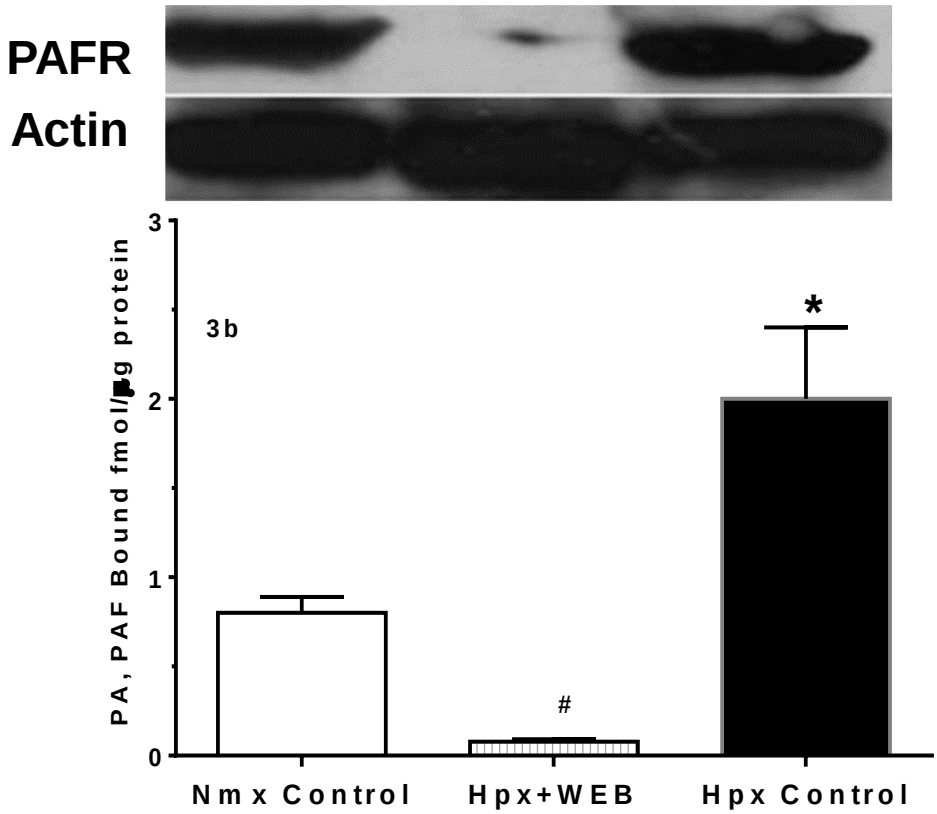

Figure 3 show the PAFR protein measured from the three groups of pups. Data are means ± SEM, n=5 and expressed as ratio of protein band to band of actin or GAPDH (for PA vessel proteins) as internal standards. PAFR protein by lung membranes of the Hpx control pups, Figure 3a, was 1.26 ± 0.054 which was greater than 0.81 ± 0.06 expressed by the Nmx control pups. Expression by Hpx+WEB lungs was 0.51 ± 0.032. Protein expression by PA membranes of the three groups of pups is shown in Figure 3b. Expression by Hpx control was 0.46 ± 0.065, which was greater than 0.31 ± 0.020 expressed by lung membranes of the Nmx control pups. Hpx+WEB group expressed 0.024 ± 0.0050. *p <0.05, different from Nmx control or Hpx+WEB. #p < 0.05, different from Nmx control.

Chronic hypoxia and expression of other signaling proteins: Nuclear factor κappa Beta (NF-kB) is a family of transcription factors that regulate a variety of cell functions.[14] We determined NF-kB p65 protein expression by the three groups of pups. We also determined TLR4 protein expression in order to establish a probable crosstalk in induction of inflammatory response or hyperplasia, *in vivo*, between NF-kB p65 signaling and TLR4 by these pups. Figure 4 shows the effect of chronic hypoxia exposure on expression of NF-kB p65, left panel, and TLR4, right panel, by the lung proteins of the neonatal pups, presented as a ratio of total actin expression. Chronic hypoxia significantly stimulated expression of NF-kB p65 by proteins of Hpx control pups, >2.5-fold more expression than Nmx control lungs. Treatment with WEB 2170 in chronic hypoxia obliterated NF-kB p65 protein expression by the Hpx+WEB pups. With respect to TLR4, lung proteins of the Hpx control pups expressed TLR4 proteins, but TLR4 protein was not detected from lungs of the Nmx control pups. Also, WEB 2170 treatment in chronic hypoxia obliterated TLR4 expression as was observed in

lung proteins of the Nmx control pups. We have demonstrated, in previous reports that PAF induces pulmonary cell growth by activating the upstream and downstream effectors of NF-kB p65 signaling cascade.[18,22] Here we show that chronic hypoxia stimulated NF-kB p65 protein expression over normoxia conditions, and stimulated TLR4 production with no effect in normoxia. The finding of a role of TLR4 in chronic hypoxia induced pulmonary hypertension is novel and the involvement PAFR in TLR4 effect under this condition is also novel and worth further exploration. A recent report in adult rats similar to our investigation, determined NF-kB p65 and TLR4 expression after 5 days of hypoxia exposure. The study measured expression of these proteins in gut environment.[33] In that report, hypoxia upregulated both NF-kB p65 and TLR4 expression, but unlike our study, TLR4 was expressed in normoxia. This difference may be due to the different organs studied: lungs in the present study versus gut in the recent report.[33] The difference may also be due to the physiological functions of the organs studied; the lung which is involved in oxygen exchange in our study versus the gastrointestinal tract involved in nutritional maintenance in the recent report.[33]

Figure 4: NF-kB p65 and TLR4 protein expression by Lungs of neonatal rat exposed to chronic hypoxia.

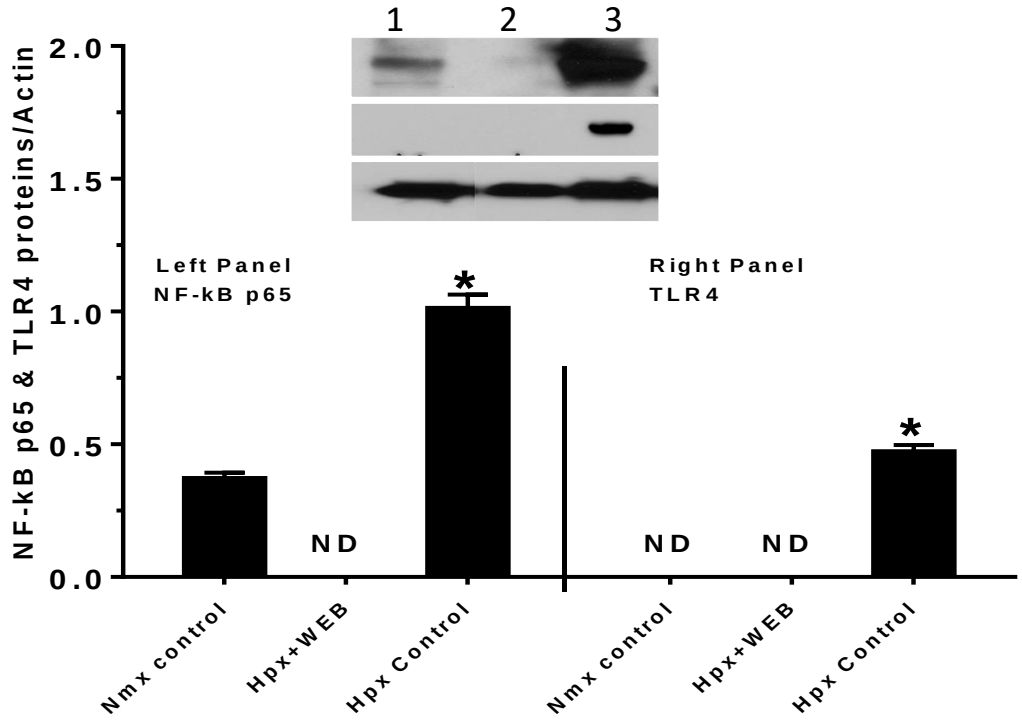

Figure 4 shows expression of the nuclear signaling proteins we studied. Data are means ± SEM, n=5 and presented as ratio of protein expressed to the actin internal standard: Inset; lane 1 Nmx control, lane 2 Hpx+WEB, lane 3 Hpx control, for both NF-kB p65 protein top row and TLR4 protein middle row. Expression of NF-kB p65 by the pups, figure 4, Left Panel in Hypoxia control (Hpx Ctrl) was 1.014 ± 0.050, which was greater than 0.373 ± 0.020 by normoxia control (Nmx Ctrl). NF-kB p65 was not detected in proteins from Hpx+WEB. For TLR4, Right Panel, expression by Hpx Ctrl pups was 0.474 ± 0.023. TLR4 protein was not detected in proteins isolated from Nmx Ctrl or Hpx+WEB pups. *p <0.05, different from Nmx control or Hpx+WEB.

In summary: Employing a chronic hypoxia regimen, we have demonstrated the involvement of PAF as an important mediator of neonatal pulmonary hypertension. PAF is an autocid, whose synthesis and degradation occur locally. It is synthesized at the site of action following an appropriate stimulus. The fact that WEB reduced PAF synthesis, PAF binding and PAFR expression strongly suggests a direct involvement of PAF in the pathology of neonatal pulmonary hypertension. Furthermore, TLR4 protein was expressed only by the Hpx Control group, suggesting that, *in vivo*, TLR4 may not play significant role in mediation of inflammatory state of neonatal lungs under normal oxygen environment. Interestingly, WEB treatment abrogated the ability of PAF to induce NF-kB p65 expression and did not stimulate TLR4 protein expression. These data show that chronic hypoxia induces PAFR expression in lungs of the pups and suggests that increased PAFR expression and PAFR binding are responsible for the right ventricular hypertrophy and pulmonary hypertension. Inhibition of PAF effects with a PAFR antagonist, WEB 2170, though it did not improve weight gain of the pups in hypoxia, prevented hypertrophy in the pups and thus the data accumulated in this study of chronic hypoxia protocol were not adversely impacted by weight of the pups.

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