Title
Effects of combined hyperoxia and cyclooxygenase inhibition in neonatal rat lungs

Permalink
https://escholarship.org/uc/item/6w78q187

Journal
American Journal of Translational Research, 2(3)

ISSN
1943-8141

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Publication Date
2010-06-08

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Indomethacin is a non-selective inhibitor of prostaglandin (PG) synthesis used prenatally for tocolysis and postnatally for the closure of a symptomatic patent ductus arteriosus (PDA). Its use is associated with significant renal, gastrointestinal and central nervous system effects [1-3]. Ibuprofen, another non-selective inhibitor of PG synthesis, is as effective as indomethacin for closure of a symptomatic PDA with fewer side effects [2,4,5]. However, postnatal ibuprofen, but not indomethacin, has been shown to cause persistent pulmonary hypertension of the newborn (PPHN) [6-10] which resolved with inhaled nitric oxide (iNO). The mechanism(s) responsible for this difference between the two drugs have not been investigated and may be due to differential effects on factors that regulate pulmonary vasomotor tone.

Pulmonary vascular circulation increases approximately 10-fold after birth secondary to lung inflation and dilatation of the pulmonary vascular bed. Control of this transition involves a dynamic interaction between pulmonary vasodilators and vasoconstrictors, such as PGs, endothelin (ET)-1, and NO. Failure of the pulmonary vessels to transition, due to disturbances in the harmonious interplay between pulmonary vasodilators and vasoconstrictors results in PPHN [11]. Administration of PG synthetase inhibitors particularly during a critical time of lung development produces changes in pulmonary vascular smooth muscle architecture and tone [12]. This effect may be mediated in part by suppression of dilator PGs which modulate the intrinsic constrictor activity of ET-1 [13]. ET-1 is a potent...
vasoconstrictor that plays a significant role in the pathophysiology of neonatal PPHN. Overexpression of its receptors, particularly ET<sub>a</sub>, was noted in the thickened pulmonary arteries of neonates who had PPHN with or without diaphragmatic hernia [14,15]. Studies have reported an association between elevated endogenous plasma ET-1 during the first few days of life and the incidence of PPHN [16-18].

Exposure to hyperoxia predisposes the preterm neonate to generation of reactive oxygen species (ROS) and oxidative injury due to immature antioxidant defense mechanisms. 8-epi-PGF<sub>2α</sub> (or 8-isoprostan) is a free radical lipid peroxidation product of arachidonic acid via a non-enzymatic pathway [19-23], and a reliable and proven biomarker for oxidative stress [19,24]. Elevated plasma 8-isoprostan has been shown to be associated with BPD and PVL in preterm infants [25]. Therefore, considering the involvement of NO and ET-1 in the development of PPHN and the role of 8-isoprostan in oxidative injury, we proposed the hypothesis that the reported effects of ibuprofen on the immature lungs are due to alterations in biochemical and molecular regulators of oxidative stress and NO signaling. Our hypothesis was tested with the following objectives: 1) to compare the effects of ibuprofen and indomethacin on biomolecular regulators of oxidative stress and NO signaling; 2) to determine whether the effects are different when exposed to normoxia or hyperoxia; and 3) to establish whether postponing treatment is more beneficial than early postnatal treatment.

Material and methods

All experiments were approved by Memorial Health Services Institutional Animal Care and Use Committee, Long Beach, CA. Animals were cared for according to the guidelines outlined by the Guide for the Care and Use of Laboratory Animals, and euthanasia was carried out according to the 2007 American Veterinary Medical Association Guidelines on Euthanasia.

Experimental design

Certified infection-free, timed-pregnant Sprague Dawley rats carrying fetuses of known gestational age (18 days) were purchased from Charles River Laboratories (Wilmington, MA). The pregnant rats were placed in USDA-compliant cages and remained undisturbed until delivery of their pups (22 days gestation). The animals were housed in an animal facility with a 12-hour-day/12-hour-night cycle and provided standard laboratory diet and water ad libitum. On the day of birth, newborn rat pups delivering on the same day were weighed, measured for linear growth (crown to rump length), pooled and randomly assigned to expanded litters of 17 pups/litter. One dam remained with the same litter for the entire study. On the day of birth, litters were randomly assigned to either room air (RA) or 50% O<sub>2</sub> (a mid range of hyperoxia). For early treatment, litters received intraperitoneal (IP) injections of: 1) indomethacin (0.2 mg/kg IP) on day 1 (P1), followed by 0.1 mg/kg on P2 and P3; 2) ibuprofen (10 mg/kg) on P1 followed by 5 mg/kg on P2 and P3; or 3) equivalent volume saline IP on P1, P2, & P3, prior to euthanasia on P4. For late treatment, litters were randomized to IP injections of: 1) indomethacin (0.2 mg/kg IP) on P4, followed by 0.1 mg/kg on P5 and P6; 2) ibuprofen (10 mg/kg) on P4 followed by 5 mg/kg on P5 and P6; or 3) equivalent volume saline IP on P4, P5, & P6, prior to euthanasia on P7. Litters assigned to hyperoxia were placed in the oxygen chamber from the first day of life until euthanasia at either P4 or P7. Indomethacin IV (Merck & Co., Inc., West Point, PA) was diluted in 0.9% saline prior to injection, and ibuprofen lysine IV solution was provided by Ovation Pharmaceuticals (Deerfield, IL) and diluted in 0.9% saline prior to injection. The doses of indomethacin and ibuprofen were based on doses currently administered to preterm neonates for symptomatic PDA. Immediately following euthanasia, the whole lungs were removed and the wet weight was determined. Tissue samples were excised from the left lower lobe, rinsed in ice-cold phosphate buffered saline (PBS) to remove blood elements and frozen immediately at -80°C for RNA isolation. Other samples were placed in 2 mL sterile normal saline and homogenized for enzyme immunoassay of 8-epi-PGF<sub>2α</sub>, 8-OHdG, ET-1, big ET-1, NO<sub>x</sub>), or placed in 10% phosphate-buffered formalin for histopathology.

Hyperoxia

On the day of birth, the litters were placed with the dams in specialized oxygen chambers (BioSpherix, New York) attached to an oxycycler. The chambers are optimized for gas efficiency and provide adequate ventilation for the ani-
mals in a controlled atmosphere with minimal gas usage. From outside the chamber, the oxy-cycler senses oxygen inside the chamber and infuses nitrogen to reduce oxygen and oxygen to raise it to maintain an environment of 50% hyperoxia throughout the experimental time. Oxygen content inside the chamber was continuously monitored and recorded on a Dell Computer. Carbon dioxide in the chamber was monitored and removed from the atmosphere by placing soda lime within the chamber. The animals remained undisturbed except during the bedding change, and food and water refill which occurred every 3 days, and lasted no more than 3 minutes.

Measurement of 8-iso-PGF$_{2\alpha}$

8-iso-PGF$_{2\alpha}$ levels in lung tissue homogenates were determined using the Direct 8-iso-PGF$_{2\alpha}$ EIA kit for use with serum, plasma, and tissue from Assay Designs, Inc (Ann Arbor, MI). Samples were first hydrolyzed with NaOH and neutralized with HCI, prior to centrifugation for 5 minutes at 14,000 rpm in a microcentrifuge and the clear supernatant was used for the assay, according to the manufacturer’s protocol. The standard curve ranged from 0 to 100,000 pg/mL. The sensitivity of the assay was 40 pg/mL and the inter- and intra-assay variations for each assay were <10%.

Measurement of 8-hydroxy-2’deoxyguanosine

8-hydroxy-2’deoxyguanosine levels in lung tissue homogenates were determined using the DNA Damage ELISA kit for the detection and quantitation of 8-OHdG, from Assay Designs, Inc. Following dilution with sample diluent samples were assayed according to the manufacturer’s protocol. The standard curve ranged from 0 to 60 ng/mL. The sensitivity of the assay was 0.59 ng/mL and the inter- and intra-assay variations for each assay were <10%.

Measurement of endothelins

ET-1 and Big ET-1 in lung homogenates were measured using TiterZyme enzyme immunoassay kits obtained from Assay Designs, Inc. according to the manufacturer’s recommendations. For big ET-1 and ET-1, the standard curves ranged from 0.78 pg/mL to 100 pg/mL. The sensitivity of the assay big ET-1 assay was 0.72 pg/mL and the sensitivity for ET-1 was 0.14. The inter- and intra-assay variations for each assay were <10%.

Measurement of NOx

Total NOx levels in lung homogenates were examined using commercially available Total NO assay kits purchased from Assay Designs, Inc., according to the manufacturer’s protocol. Briefly, samples were diluted in reaction buffer and nitrate reductase was added to the sample to convert nitrate to nitrite. Nitrite was determined using the Griess Reagent. The standard curve ranged from 0 to 100 mmole/L and the inter- and intra-assay variations were <10%.

Total cellular protein assay

On the day of the assay, the lung homogenates were assayed for total cellular protein using the dye-binding Bio-Rad protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. The standard curve was linear from 0.05 to 1.45 mg/ml of protein.

Histology

At the time of euthanasia, total lung wet weight was determined and tissue from the left lobe as fixed with 10% phosphate-buffered formalin for at least 24 hours. After fixation, the samples were cut into 5 pieces of equal thickness and taken to Memorial Medical Center Pathology laboratory for processing and staining according to standard laboratory techniques for a total of 5 blocks per group. Digital images (20X magnification) were taken using Spot software version 4.6 (Diagnostic Instruments, Sterling Heights, MI), in combination with a high resolution Spot RT3 camera (Diagnostic Instruments), Olympus BH-2 microscope (Center Valley, PA) and Dell Optiplex GX280 computer (Dell Computer Corporation, Dallas, TX).

Real-time polymerase chain reaction

At euthanasia, tissue from the left lobe was frozen immediately for RNA isolation. Total RNA was extracted using RNA Pro solution (MP Bio, Solon, Ohio) and allowed to digest for 5 minutes at room temperature. The samples were transferred to microtubes containing ceramic beads and placed in a FastPrep-24 instrument (MP Bio) for 40 seconds. The samples were centrifuged at 13,000 rpm at 4°C for 5 minutes, and the supernatant transferred to a clean eppendorf tube. After addi-
tion of chloroform, the samples were vortexed for 30 seconds and centrifuged at 13,000 rpm at 4°C for 5 minutes. The upper aqueous phase was transferred to a clean eppendorf tube containing 0.5 mL of ice-cold 100% ethanol. The samples were placed in a -20°C freezer for 60 minutes to precipitate the RNA. Following precipitation, the samples were centrifuged at 13,000 rpm at 4°C for 20 minutes and the resulting pellet was washed in 75% ethanol/water and redissolved in 100 μL nuclease-free water. The amount of RNA was quantified at 260 nm using a Beckman spectrophotometer and diluted to 1 mg/mL. Cleanup of the RNA was performed using RNEasy mini cleanup kits (Qiagen) followed by on-column treatment with DNase I (Qiagen). Reverse transcriptase was performed using RT² Profiler Rat NO Signaling PCR Array System (a pathway focused gene expression profiler) from SABiosciences, Frederick, MD. The real-time PCR arrays were carried out in duplicate on 96-well plates pre-coated with 84 genes regulating NO signaling.

Statistical analysis

Unpaired t-tests or Mann Whitney U tests were used to determine differences between RA and hyperoxia groups, or early versus late effects, following Levene’s test for equality of variances. One-way analysis of variance was used to determine differences among the treatment groups for normally-distributed data, and Kruskal-Wallis test was used for non-normally-distributed data following Bartlett’s test for equality of variances. Post hoc analysis was performed using the Tukey and Student-Newman-Keuls test for significance, as appropriate. Significance was set at p<0.05 and data are reported as mean ± SEM. All analyses were two-tailed and performed using SPSS software version 16.0 (SPSS Inc., Chicago, IL) and GraphPad Prism software version 5.02 (GraphPad Inc., San Diego, CA).

Results

Effects on growth

Percentage change in body weight (calculated as: total body weight in grams, at euthanasia - total body weight at birth/total body weight at birth x 100) were calculated to determine effects of the drug on growth (data not shown). For the early treatment group, percentage change in body weight was not different between the RA and hyperoxia groups treated with saline and ibuprofen, but was significantly elevated with indomethacin treatment in hyperoxia (48.3±3.5%) versus RA (31.7±5.5%, p<0.05), as well as saline (36.7±2.8%, p<0.05) and ibuprofen (33.6±3.4%, p<0.01) treatment in hyperoxia. In the late treatment groups, hyperoxia resulted in decreased weight gain with saline treatment (71.7±3.5%, p<0.001) compared to RA (103.1±4.4%). This effect appeared to be reversed in the indomethacin-treated group exposed to hyperoxia (110.7±9.0%, p<0.01). Treatment with ibuprofen suppressed weight gain in RA (73.5±7.6%, p<0.001) compared to saline in RA. A similar but diminished effect was noted with indomethacin treatment in RA (82.5±5.1%, p<0.05) compared to saline in RA and indomethacin in hyperoxia (p<0.01). Lung to body weight ratios were comparable among the early and late treatment groups, and were not affected by hyperoxia.

Effect on oxidative stress

As expected, lung tissue 8-iso-PGF2α levels (pg/mg protein) were similarly elevated in all groups exposed to hyperoxia with early (saline:4096.2±566.1; indomethacin :4485.7±846.0; ibuprofen:3012.2±279.1; p<0.001) and late (saline:4022.5±288.1; indomethacin :6592.2±535.9; ibuprofen:4957.8±488.5, p<0.001) treatment compared to the early (saline:1329.7±125.1; indomethacin:1921.6±183.5; ibuprofen:870.3±197.4) and late (saline:859.4±250.5; indomethacin :1720.8±101.1; ibuprofen:1175.9±64.3) RA groups (Figure 1). 8-iso-PGF2α levels were significantly higher with early indomethacin treatment in RA than saline (p<0.05) and ibuprofen (p<0.01) in RA. A similar response was noted with late indomethacin treatment in RA compared to saline (p<0.05) and ibuprofen (p<0.01) in RA. A similar response was noted with late indomethacin treatment in RA compared to saline (p<0.05) and ibuprofen (p<0.01) in RA. A similar response was noted with late indomethacin treatment in RA compared to saline (p<0.05) and ibuprofen (p<0.01) in RA.

Effect on DNA damage

Early treatment with both indomethacin (8.2±0.5, p<0.05) and ibuprofen (8.6±1.1, p<0.05) reduced lung 8-OHdG levels (ng/mg protein) in hyperoxia compared to saline-treated littermates in hyperoxia (12.1±1.3). The effect was also significantly different from indomethacin in RA (11.0±1.1) or ibuprofen in RA (13.0±2.2). For late treatment, only indomethacin effectively...
**Hyperoxia, cyclooxygenase inhibition & neonatal rat lungs**

Decreased lung 8-OHdG levels in hyperoxia (6.5±0.7, p<0.05) compared to indomethacin RA (13.3±2.5), saline hyperoxia (15.3±1.5), and ibuprofen hyperoxia (14.1±3.2) as demonstrated in Figure 2.

**Effect on nitric oxide metabolites (NOx)**

Early treatment with ibuprofen elevated NOx levels (mmol/mg protein) in RA (29.1±2.9) compared to ibuprofen treatment in hyperoxia (16.8±3.1, p<0.01) and indomethacin treatment in RA (14.5±3.6, p<0.05). Similar elevations were noted with indomethacin treatment in hyperoxia (25.3±3.4, p<0.05) compared to indomethacin treatment in RA. For late treatment, lung NOx levels were significantly elevated in the saline hyperoxia group (34.8±5.8, p<0.05) compared to saline RA (18.4±3.4) and in the indomethacin hyperoxia (38.6±5.6, p<0.01) compared to indomethacin RA (22.4±1.4) groups. No elevations were noted with ibuprofen (Figure 3).

**Effect on big ET-1**

Figure 4 shows that in the early treatment groups, ibuprofen increased big ET-1 levels (pg/mg protein) in RA (3.91±0.88) compared to saline RA (1.77±0.3, p<0.05) and indomethacin in RA (1.15±0.31, p<0.01). In contrast, ibuprofen treatment in hyperoxia decreased big ET-1 levels (1.07±0.28) compared to saline in hyperoxia (2.08±0.31, p<0.05) and ibuprofen in RA (p<0.01). A similar reduction in big ET-1 levels was noted with indomethacin treatment in hy-
peroxia (0.9±0.18, p<0.05) compared to saline treatment in hyperoxia (2.08±0.32). Lung big ET-1 levels were decreased in all late treatment groups in hyperoxia (saline:1.02±0.28, p<0.01; indomethacin :0.83±0.13, p<0.001; ibuprofen:1.04±0.17, p<0.05) compared to RA (saline:3.33±0.44; indomethacin :3.0±0.39; ibuprofen:1.92±0.3).
Effect on ET-1

Treatment with early indomethacin resulted in higher lung ET-1 levels (pg/mg protein) in RA (2.0±0.3, p<0.05) compared to saline in RA (0.93±0.19) and ibuprofen in RA (0.79±0.12).
as shown in Figure 5. Treatment with saline in hyperoxia resulted in higher ET-1 levels (1.9±0.24, p<0.05) compared to saline in RA (0.93±0.19), indomethacin in hyperoxia (0.94±0.23, p<0.05), and ibuprofen in hyperoxia (0.43±0.06, p<0.01). In the late treatment groups, indomethacin treatment in RA caused similar elevations in ET-1 levels (2.2±0.33, p<0.001) compared to indomethacin in hyperoxia (0.33±0.12), saline in RA (0.65±0.15) and ibuprofen in RA (0.78±0.12).

**Histology**

Histological analysis showed that early treatment with ibuprofen and to a greater extent, indomethacin resulted in increased alveolar size and reduction in the appearance of secondary crests (Figure 6). These characteristics were sustained with late treatment (Figure 7).

**Profile of genes regulating NO signaling and oxidative stress**

Following early treatment, hyperoxia alone resulted in downregulation of a majority of the genes of interest that are involved in regulation of NO signaling except nNOS, and oxidative stress. Of note, indomethacin treatment in RA resulted in upregulation of all the GPX and SOD genes (Table 1). Following late treatment, a reversal of gene expression was noted in the group exposed to hyperoxia. Most notably were the 6-fold downregulation with saline treatment, and the 3-fold increased expression with indomethacin treatment of nNOS compared to RA controls. Increased expression of most genes involved in regulation of oxidative stress was observed with late treatment. However, a 32-fold decrease in GPX2 was noted in the salinel-treated hyperoxia group, as well as a 106-fold downregulation in GPX7 in the ibuprofen-treated hyperoxia group and an 8-fold, and a 60-fold upregulation in GPX5 and 6, respectively, in the indomethacin-treated hyperoxia group (Table 2).

**Discussion**

To examine our hypothesis that the reported effects of ibuprofen on the immature lungs are due to alterations in biochemical and molecular regulators of oxidative stress and NO signaling,

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The data are presented as fold change from Sal-RA-E. All data were corrected using 5 different housekeeping genes. Genes are selected from a profile of 84 genes. In alphabetical order the genes of interest are: CALM1, calmodulin 1; CAT, catalase; CAV-1, caveolin-1; eNOS, endothelial nitric oxide synthase; GPX1-7, glutathione peroxidase 1-7; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; SOD1,2, superoxide dismutase 1,2.
we raised three questions: 1) do the effects of ibuprofen and indomethacin on biomolecular regulators of oxidative stress and NO signaling differ despite their similar action as non-selective COX inhibitors? 2) are the effects of ibuprofen and indomethacin different when administered in normoxia and hyperoxia (50% O₂)?; and 3) does postponing treatment of ibuprofen and indomethacin to a later maturational age result in more beneficial effects? To address these questions, we first measured pulmonary levels of 8-epi-PGF$_{2\alpha}$ which is a free radical lipid peroxidation product of arachidonic acid via a non-enzymatic pathway [19-23], and a reliable and proven biomarker for oxidative stress [19,24]. We also measured 8-OHdG, a reliable biomarker for oxidative DNA damage [26-30]. As expected, 8-epi-PGF$_{2\alpha}$ was higher in the lungs of hyperoxic rats, reflecting oxidative stress, and treatment with both drugs did not significantly ameliorate this response, confirming a non-enzymatic ROS pathway. However, treatment with early ibuprofen tended to lower oxidative stress in contrast to early and late indomethacin. This may suggest that a portion of the isoprostane may be COX-derived. Surprisingly, we found that the levels of 8-epi-PGF$_{2\alpha}$ were increasingly higher with indomethacin treatment and was most evident with late treatment. Whether this is due to a shift in arachidonic acid (AA) to the non-enzymatic ROS pathway due to inhibition of COX, remains to be determined.

Hyperoxia results in the production of ROS such as superoxide anion and hydroxyl radical. ROS causes oxidation of protein, DNA and lipids and initiate inflammatory responses in the lungs. Regulators of ROS include catalase (CAT), glutathione peroxidase (GPX) and superoxide dismutase (SOD). In the lungs, GPX content is approximately 140 times higher than the circulation [31], suggesting a key role in antioxidant defense of the lungs. SODs are the only enzymatic system-decomposing superoxide radicals and are important for oxidative stress [31]. In our study, early treatment with indomethacin in RA resulted in a 2.5-fold decrease in CAT, while GPX1-7 and SOD2 were either upregulated or remained unchanged from controls, as well as a

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Table 2. Real-time polymerase chain reaction profile of genes involved in nitric oxide signaling and oxidative stress following late ibuprofen and indomethacin treatment in room air or 50% oxygen

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<td>-1.8</td>
<td>2.3</td>
<td>-1.0</td>
<td>59.5</td>
</tr>
<tr>
<td>GPX7</td>
<td>1.3</td>
<td>1.1</td>
<td>-106.3</td>
<td>-1.1</td>
<td>-1.4</td>
</tr>
<tr>
<td>SOD1</td>
<td>1.3</td>
<td>-1.1</td>
<td>1.1</td>
<td>-1.1</td>
<td>-1.1</td>
</tr>
<tr>
<td>SOD2</td>
<td>1.6</td>
<td>1.2</td>
<td>1.2</td>
<td>1.3</td>
<td>-1.0</td>
</tr>
</tbody>
</table>

The data are presented as fold expression difference compared to Sal-RA-E. All data were corrected using 5 different housekeeping genes. Genes are selected from a profile of 84 genes. Downregulation is represented by parentheses and upregulation is represented by no parentheses. Genes of interest are as presented in Table 1.
robust upregulation in GPX5 and 6 with late treatment in 50% O2. This may suggest a compensatory response to oxidant/antioxidant imbalance. We also found that GPX2 was the only isoform of pulmonary GPX that responded after 6 consecutive days of hyperoxia with a marked (32-fold) reduction in gene expression. Increasing evidence suggest a role for GPX2 in protecting the lungs against hyperoxic injury [32]. This finding may suggest inability of the immature lungs to mount a defense against increasing levels of ROS. GPX7 was markedly downregulated (106-fold) with late ibuprofen treatment in hyperoxia. Our understanding of the mechanism for decreased GPX7 with ibuprofen is limited and requires further study. Interestingly, GPX5 (8-fold) and to a greater extent, GPX6 (60-fold) was abundantly expressed with late indomethacin in hyperoxia. This may be a compensatory effect resulting from the increasing levels of 8-epi-PGF2α in that group. GPX6 has been shown to be upregulated 30-fold in response to sodium nitroprusside (a nitric oxide donor) in legumes [33]. Alternatively, high GPX6 expression in response to late indomethacin treatment in hyperoxia may be due higher ROS and higher NO production in that group. Our data support a role for GPX6 in redox sensing and regulation in the hyperoxic lungs.

Hyperoxic DNA damage results from the interaction of ROS with DNA, lipids and proteins, cellular targets for oxidative damage. 8-OHdG is a modified nucleoside base which is a by-product of DNA damage. It is widely used as a biomarker for oxidative DNA damage because of its ability to cause guanine to thymine transitions in DNA, its mutagenicity, and it is perceived by the cell as a threatening lesion that has to be removed rapidly [28]. We were surprised to find a lack of increase in 8-OHdG in the hyperoxia-exposed, early saline-treated lungs, and only a trend for higher levels in the late saline-treated lungs, despite clear evidence of oxidative stress. This may be due to insufficient exposure time to cause oxidative DNA damage. Interestingly, both ibuprofen and indomethacin suppressed pulmonary 8-OHdG during early treatment in hyperoxia, but had no effect in normoxia. This may suggest an involvement of PGs in oxidative stress. However, since only indomethacin maintained the suppressive effect during late treatment, we suggest that indomethacin may be more effective and longer-lasting than ibuprofen. Postponing treatment of ibuprofen and indomethacin had no beneficial effects on markers for oxidative DNA damage.

NO is produced by nitric oxide synthase (NOS) and plays a major role in lung development [34], maintenance of alveolar epithelial cell phenotype [35] and vasomotor tone [36]. All three NOS isoforms (eNOS, iNOS and nNOS) are present in the lungs and their expression and NO production are increased with lung maturation. We found that pulmonary NOx was increased with early and late indomethacin treatment in hyperoxia, early ibuprofen in RA, and late saline in hyperoxia. However, we did not find any appreciable effects with ibuprofen treatment in hyperoxia. NO is also a free radical that is upregulated in hyperoxia and results in vasodilation [37], the lack of response noted with ibuprofen treatment may explain at least in part, the transient PPHN noted in some infants exposed to ibuprofen for symptomatic PDA, an effect that was resolved with inhaled NO [6,7,9]. The difference in response to hyperoxia-induced NO upregulation between the two NSAIDs may be due to differences in their pharmacologic characteristics and selectivity for COX-2 which interacts with NO synthase [39]. Gene profile of NO signaling genes during early treatment showed that while some genes are upregulated and some were downregulated, increased NOx in the ibuprofen RA group was associated with upregulation of iNOS and nNOS and increased NOx in the indomethacin O2 group was associated with iNOS. This suggests that iNOS is most likely the source of pulmonary NO in this setting. Conversely, NOx elevation with late saline in hyperoxia was associated with eNOS upregulation while NOx elevation in the late indomethacin hyperoxia group was associated with nNOS upregulation. Together these findings suggest that hyperoxia-induced NO is generated from eNOS, whereas NO generated from ibuprofen and indomethacin, whether in RA or hyperoxia is predominantly from iNOS.

ETs are potent vasoactive mediators that are produced by the vascular endothelium. NO interacts with ET-1 by either blocking the conversion of big ET-1 to ET-1, or diffusing to the smooth muscle cell and activating cGMP to produce vasodilation [39-41]. Our data showed that both early and late indomethacin and ibuprofen suppressed pulmonary big ET-1 levels in hyperoxia, suggesting either increased conversion to ET-1 or decreased production. However, since ET-1...
was also decreased in the hyperoxia-exposed groups it is unlikely that these effects are due to increased conversion. Although ibuprofen blunted the hyperoxia-induced NO response, we did not observe a compensatory increase in ET-1. In fact, early treatment with ibuprofen suppressed pulmonary ET-1 levels. This may be due to an indirect effect through suppression of PGs, since an interaction between ET-1 and COX-2 has been demonstrated [42]. Other studies have shown that ET-1 is a potent agonist for AA release and PG synthesis [43]. Our findings that ibuprofen regulates ET-1 levels corroborate those previous reports.

The most interesting finding was the effect of indomethacin and ibuprofen on the pulmonary architecture which was consistent despite the timing of treatment or the atmospheric oxygen environment. The rats were exposed to treatment at a critical time when the alveoli are being formed by septation of the large saccules, which is approximately the 3rd to 4th postnatal day and completed by the 2nd postnatal week [44]. Inhibition of septation produce larger and fewer alveoli, an effect that appears to be permanent, persisting until adulthood [45]. Our findings are consistent with and confirm an earlier study which showed that indomethacin treatment resulted in increased alveolar size, suggesting a role for PGs in alveolar formation [46]. Our findings are also consistent with a previous report of increased alveolar surface area with ibuprofen treatment in ventilated premature baboons who had improved pulmonary mechanics [34], further implicating a key role for PGs in alveolar development.

In conclusion, we found that ibuprofen and indomethacin treatment resulted in similar lung architecture when administered in RA or hyperoxia suggesting an involvement of PGs in lung development. Both drugs had no beneficial effects on oxidative stress. Indomethacin treatment resulted in higher pulmonary levels of 8-epi-PGF2α which was associated with overexpression of GPX6 with late treatment suggesting a role in redox sensing and regulation in the hyperoxic lungs. Postponing treatment had no significant beneficial effects on biomolecular regulators of oxidative stress and NO signaling. We therefore speculate that suppression of hyperoxia-induced NOX production with early and late ibuprofen treatment may explain in part, the transient PPHN seen in ibuprofen-treated preterm infants.

Acknowledgement

The authors thank the technicians in the Pathology Department of Long Beach Memorial Medical Center for their participation in processing and staining the lung tissue samples.

This work was made possible through grants from the Memorial Medical Center Foundation, Long Beach, CA; Ovation Pharmaceuticals, Deerfield, IL; and Ikaria, Clinton NJ.

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References

[8] Levin DL, Mills LJ, Weinberg AG. Hemodynamic, pulmonary vascular, and myocardial abnormalities secondary to pharmacologic constriction of


