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Cyclooxygenase-2 in Newborn Hyperoxic Lung Injury

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Abstract

Supraphysiological O₂ concentrations, mechanical ventilation, and inflammation significantly contribute to the development of bronchopulmonary dysplasia (BPD). Exposure of newborn mice to hyperoxia causes inflammation and impaired alveolarization similar to that seen in infants with BPD. Previously, we demonstrated that pulmonary cyclooxygenase-2 (COX-2) protein expression is increased in hyperoxia-exposed newborn mice. The present studies were designed to define the role of COX-2 in newborn hyperoxic lung injury. We tested the hypothesis that attenuation of COX-2 activity would reduce hyperoxia-induced inflammation and improve alveolarization. Newborn C3H/HeN mice were injected daily with vehicle, aspirin (non-selective COX-2 inhibitor), or celecoxib (selective COX-2 inhibitor) for the first 7 days of life. Additional studies utilized wild type (C57Bl/6, COX-2^{+/+}), heterozygous (COX-2^{+/-}), and homozygous (COX-2^{-/-}) transgenic mice. Mice were exposed to room air (21% O₂) or hyperoxia (85% O₂) for 14 days. Aspirin-injected and COX-2^{-/-} pups had reduced levels of monocyte chemoattractant protein (MCP-1) in bronchoalveolar lavage fluid (BAL). Both aspirin and celecoxib treatment reduced macrophage numbers in the alveolar walls and airspaces. Aspirin and celecoxib treatment attenuated hyperoxia-induced COX activity, including altered levels of prostaglandin (PG)D₂ metabolites. Decreased COX activity, however, did not prevent hyperoxia-induced lung developmental deficits. Our data suggests that increased COX-2 activity may contribute to pro-inflammatory responses, including macrophage chemotaxis, during exposure to hyperoxia. Modulation of COX-2 activity may be a useful therapeutic target to limit hyperoxia-induced inflammation in preterm infants at risk of developing BPD.

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Keywords

Bronchopulmonary dysplasia; Hyperoxia; Cyclooxygenase-2; Prostaglandins

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Introduction

Preterm infants are born with immature lungs and frequently require respiratory support. Although necessary to maintain adequate oxygenation, hyperoxia exposure contributes to the development of chronic lung disease in infancy also known as bronchopulmonary dysplasia (BPD) [1]. Currently, BPD is defined as requiring supplemental O₂ for >28 days of life and/or 36 weeks' corrected gestational age [2, 3]. Pathologically, BPD is characterized by impaired alveolar and vascular development [1]. While preterm infant mortality has decreased over the past 20 years, the incidence of BPD is relatively unchanged [4].

Perinatal inflammation, originating from multiple sources including *in utero* infection, hyperoxia, mechanical ventilation, and pulmonary infections [5], contribute to the development of BPD [6, 7]. Preterm infants at risk of developing BPD have increased expression of many pro-inflammatory mediators including interleukin (IL)-6, IL-8, IL-1 β , and IL-10 [8]. Multiple studies have reported increased levels of leukocyte and pro-inflammatory chemoattractants in the lungs of preterm infants that develop BPD [9–14]. Currently, there are no effective therapies to limit inflammation in preterm infants who are at risk of developing BPD.

Cyclooxygenase (COX)-1 and its isoform COX-2 enzymatically metabolize arachidonic acid into prostaglandin (PG)H₂. Subsequently, PGH₂ becomes a substrate for synthases that metabolize PGH₂ into prostaglandins, which are bioactive lipid mediators. There is evidence of increased prostaglandin levels in preterm infants at risk of developing BPD [15–17] and increased COX activity in lung tissues of newborn mice exposed to hyperoxia [18]. Immunohistochemical analysis of the developing human lung found COX-2 expression in the bronchiolar epithelium of preterm infants who developed BPD [19]. Prostaglandins including PGD₂, PGE₂, and thromboxane (TX)B₂ have been shown to regulate multiple inflammatory processes in the lung including leukocyte chemotaxis, airway and vascular tone, and vascular permeability [20, 21].

Hyperoxia exposure, in newborn mice, causes inflammation and alveolar development deficits similar to those seen in infants with BPD [18, 22–24]. Although COX-2 expression and activity is increased in lung tissues of hyperoxia-exposed newborn mice [18], the role of COX-2 and subsequent metabolites during newborn hyperoxic lung injury remains less defined. In the present studies, we tested the hypothesis that attenuation of COX-2 activity would reduce hyperoxia-induced inflammation and subsequently protect against hyperoxia-induced lung developmental arrest in newborn mice. Newborn C3H/HeN mice were injected daily with vehicle, aspirin, a non-selective COX-2 inhibitor, or celecoxib, a selective COX-2 inhibitor. Additional studies investigated COX-2^{+/+}, COX-2^{+/-}, and COX-2^{-/-} transgenic mice. These mice express a Tyr385Phe mutation, resulting in loss of cyclooxygenase activity but preservation of peroxidase activity [25]. Mice were exposed to room air (21%

O₂) or hyperoxia (85% O₂) for 14 days. Our findings suggest that COX-2 has a pro-inflammatory role in newborn mice exposed to hyperoxia, with specific effects on chemokine production, macrophage chemotaxis, and prostaglandin levels.

Methods

Animal model

Protocols for mouse studies were approved by the Institutional Animal Care and Use Committee at Nationwide Children's Hospital, Columbus, OH and all mice were handled following National Institutes of Health guidelines. Two litters of C3H/HeN mice were matched and within 16 h of birth, one litter of pups was placed to room air (21% O₂) while the corresponding litter was placed in hyperoxia (85% O₂) for 14 days. Beginning on day 1, pups were injected daily with 40 mg/kg aspirin (Sigma-Aldrich, St. Louis, MO), 5 mg/kg celecoxib (Sigma-Aldrich), or an equal volume of vehicle (PBS). Similarly, newborn C57Bl/6 wild type (WT), heterozygous (COX-2^{+/-}), and homozygous (COX-2^{-/-}) COX-2 transgenic mice (Jackson Laboratory, Bar Harbor, ME) were exposed to 21% or 85% O₂ for 14 days. To avoid oxygen toxicity, nursing dams were rotated daily between the 21% and 85% O₂ paired litters every 24 hours. After 14 days of exposure, pups were injected with 200 mg/kg ketamine and 20 mg/kg xylazine to achieve terminal anesthesia. Lungs were harvested, lavaged, or inflation fixed. To collect bronchoalveolar lavage fluid (BAL), lungs were flushed 3× with sterile PBS. Lavage fluid was centrifuged at 3000 rpm for 10 minutes and supernatant was recovered and stored at -80°C. Lung tissues were snap-frozen and stored at -80°C.

Western blot

Protein concentrations were determined in tissue homogenates by Bradford assay. Equal amounts of protein were loaded and separated by SDS-PAGE and transferred to PVDF or nitrocellulose membranes. Following blocking, blots were probed with primary antibodies for COX-1 (rabbit polyclonal, 1:1000, Cayman, Ann Arbor, MI), COX-2 (rabbit monoclonal, 1:200, Abcam, Cambridge, MA), hematopoietic PGD synthase (rabbit polyclonal, 1:750, Cayman), and microsomal PGE synthase (rabbit polyclonal, 1:1000, Cayman). For loading control, β -actin (rabbit monoclonal, 1:10000, Abcam) primary antibody was used. Horseradish peroxidase conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (1:12000, BioRad Laboratories, Hercules, CA) were applied for 1 hour. Immunoblots were developed using enhanced chemiluminescence western blotting detection (GE Healthcare, Buckinghamshire, UK) and band densities were quantified using Image Quant TL software, version 5.0 (GE Healthcare). During band quantification, background was subtracted.

Morphometric and immunohistochemical analysis

Lungs were inflation fixed with formalin at 25 cm H₂O and embedded in paraffin. To assess alveolarization, lung sections were stained with H&E and five non-overlapping, representative microphotographs were taken at 100× magnification by an investigator blinded to group assignment. Average alveolar number, area, and perimeter were quantified using Image Pro Plus 6.3 (Media Cybernetics, Silver Spring, MD). In additional studies,

lung sections were immunohistochemically stained with an antibody specific for macrophages, F4/80 (rat monoclonal, 1:100, AbDSerotec, Raleigh, NC), hematopoietic PGD synthase (rabbit polyclonal, 1:500, Cayman), and microsomal PGE synthase (rabbit polyclonal, 1:250, Cayman). The number of macrophages was quantified on five representative microphotographs at 100× magnification per section and manually counted by an investigator blinded to group assignment.

ELISA

Keratinocyte-derived chemokine (KC) and monocyte chemoattractant protein-1 (MCP-1) levels in BAL samples were assessed by ELISA (Duoset ELISA kits, R&D systems, Minneapolis, MN) according to manufacturer protocols. Proteins levels were determined by measuring absorbance at 450 nm using a spectrophotometer, SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). Standard curves were utilized to determine chemokine concentrations.

Prostaglandin levels

Prostaglandin levels were measured in lung tissues as previously described [18]. Lung tissues were homogenized in 0.1 M NaH₂PO₄, 0.9% NaCl buffer at pH 5. An internal standard containing 0.5 ng/μL each, of deuterated PGF_{2α}, TXB₂, PGD₂, leukotriene B₄, and 5-hydroxyeicosatetraenoic acid was added to each sample. Homogenized lung tissue was immediately added to 4× sample volume 2:1 chloroform/methanol, mixed, and centrifuged at 2000 rpm for 2 min. The organic phase was extracted and placed under a stream of N₂. The chloroform/methanol extraction step was repeated and the organic phases combined. Following evaporation of the organics, lipids were reconstituted in 100 μL ethanol and analyzed by LC-MS/MS. Standard curves were used for quantification.

Statistics

Data were analyzed by unpaired student's t test, two-way ANOVA followed by Newman-Keuls multiple comparisons test, or log-rank (Mantel-Cox) test using GraphPad Prism 6.0 (GraphPad, La Jolla, CA). Statistical differences are indicated by p<0.05.

Results

COX Protein Expression in lung tissues

Pulmonary COX-2 and COX-1 protein expression was measured in lung homogenates obtained from 21% or 85% O₂-exposed pups by Western blot. COX-2 but not COX-1 expression was greater in hyperoxia-exposed pups than in room air-exposed controls (Figure 1).

Mortality and Body weights

During exposure to 21% O₂ or 85% O₂, vehicle or aspirin injected pups had a survival rate of 92–100% (Table 1). The survival rate of 21% O₂, celecoxib-injected pups was 85% but was not significantly different from 21% O₂-exposed, vehicle-injected pups. Hyperoxia-exposed pups injected with celecoxib had a survival rate of 96%. There were no significant

differences in body weight between 21% and 85% O₂ -exposed, vehicle-injected pups. Hyperoxia-exposed, aspirin-injected pups had significantly lower body weights than 21% O₂-exposed, vehicle-injected pups (Table 1).

Similarly, wild type and COX-2^{+/-} mice had a survival rate of 95–100% during exposure to 21% O₂ and 85% O₂ (Table 2). However, there was significant neonatal mortality in COX-2^{-/-} regardless of exposure. Within the first 48 h of life, 43% of 21% O₂-exposed COX-2^{-/-} and 47% of hyperoxia-exposed COX-2^{-/-} pups died (Table 2). There was no observed mortality in COX-2^{-/-} after day 3.

Bronchoalveolar lavage protein levels

Hyperoxia exposure significantly increased BAL protein concentration in the vehicle treatment group compared to all 21% O₂-exposed groups (Figure 2A). The 85% O₂ -exposed aspirin and celecoxib treatment groups were not different than the 85% O₂-vehicle treatment group or any of the 21% O₂-exposed groups. Hyperoxia-exposed WT pups had significantly higher BAL protein concentrations than 21% O₂-exposed, COX-2^{-/-} pups (Figure 2B).

KC and MCP-1 Expression in BAL

Hyperoxia exposure significantly increased BAL KC and MCP-1 protein levels. Aspirin treatment further increased KC expression in BAL of 85% O₂ -exposed mice; however MCP-1 levels were not affected. Celecoxib had no effect on hyperoxia-induced KC or MCP-1 expression (Figure 3A). In contrast, 85% O₂ -exposed COX-2^{-/-} pups had reduced KC and MCP-1 levels in BAL compared to 85% O₂ -exposed WT pups (Figure 3B).

Macrophage counts

Lung sections were stained with the macrophage surface marker, F4/80. Hyperoxia-exposed, vehicle-treated WT pups had greater numbers of F4/80+ cells per high power field (h.p.f.) than any group of 21% O₂-exposed pups (Figure 4A). Aspirin or celecoxib treatment prevented the 85% O₂ -induced influx of positive cells. Similarly, hyperoxia-exposed COX-2^{-/-} pups also had fewer F4/80 positive cells in the alveolar space compared to hyperoxia-exposed WT pups (Figure 4B).

Morphometric analysis

Alveolarization was assessed using H&E-stained lung sections. All 85% O₂ -exposed pups had significant deficits in alveolar development (Figure 5). Alveolar numbers were lower and alveolar area greater in all 85% O₂ -exposed treatment groups than in 21%O₂-exposed controls (Figure 5A). Hyperoxic exposure also decreased alveolar numbers and increased alveolar areas in WT, COX-2^{+/-}, and COX-2^{-/-} mice compared to 21% O₂-exposed controls (Figure 5B). Additionally, 21% O₂ exposed, COX-2^{+/-} pups had significantly lower alveolar numbers, compared to 21% O₂ exposed, WT pups (Figure 5B).

Prostaglandin levels

As an indirect measurement of COX activity, we assessed the effect of aspirin and celecoxib on prostaglandin levels in lung tissues of 21% O₂ and 85% O₂-exposed mice (Figure 6).

Significant differences were observed between 21% and 85% O₂ exposures in 15-deoxy-12, 14-PGJ₂, 13, 14-dihydro-15-keto PGD₂, and PGE₂. Compared to 21% O₂-vehicle injected mice, aspirin-injection significantly reduced levels of PGJ₂, PGE₂, and TBX₂. During 85% O₂ exposure, aspirin-injection reduced lung tissue levels of PGD₂, 15-deoxy-12, 14-PGJ₂, 13, 14-dihydro-15-keto PGD₂, PGE₂, and TBX₂ compared to vehicle-injected pups. Injections with celecoxib significantly reduced levels of 15-deoxy-12, 14-PGJ₂, 13, 14-dihydro-15-keto PGD₂, PGE₂, and TBX₂ in O₂ exposed pups compared to vehicle-injected, hyperoxia-exposed pups (Figure 7A). In contrast to aspirin-injection, celecoxib did not affect prostaglandin levels in 21% O₂ pups.

The increases in prostaglandin levels in 85% O₂-exposed mice compared to 21% O₂-exposed mice previously observed in the C3H/HeN vehicle-treated mice were not seen in the C57/Bl6 WT mice exposed to 85% and 21% O₂, respectively. Furthermore, there were no differences in prostaglandin levels between WT and COX-2 transgenic mice although the levels in the transgenic mice tended to be lower (Figure 7B).

Expression of prostaglandin D and E synthases

Exposure to hyperoxia led to significant increases in PGD₂ and PGE₂ and their metabolites in C3H/HeN pups (Figure 7A). To explore the mechanism responsible for these increases we measured hematopoietic PGD synthase (HPGDS) and microsomal prostaglandin E synthase (MPGES) protein levels in lung homogenates from C3H/HeN pups exposed to 21% or 85% O₂ for 14 days. Hyperoxia-exposed pups had significantly higher HPGDS and MPGES levels than 21% O₂ controls (Figure 8A and 8B).

Immunohistochemical analysis was performed to determine localization of HPGDS and MPGES within the lung. We found that only cells in the airway epithelium expressed HPGDS (Figure 8C). Expression of MPGES was detected in the cells in the alveolar walls and airspace (Figure 8C).

Discussion

In the present studies, our goal was to assess the role of COX-2 in a model of newborn hyperoxic lung injury. Similar to previous studies [18], we found that exposure to 85% O₂ for 14 days increased COX-2 expression and activity compared to 21% O₂ exposed pups (Figure 1 and 7A). Mass spectrometric measurements revealed effects of COX inhibition on prostaglandin formation including the PGD₂ metabolic pathway in lung tissues (Figure 7A). Attenuation of COX activity was associated with reduced markers of inflammation including MCP-1 expression (Figure 3) and macrophage infiltration (Figure 4). Our data suggest that COX-2 may contribute to macrophage infiltration during newborn hyperoxic lung injury.

Daily dosing of newborn mice with vehicle or aspirin had no significant effect on mortality. However, the pups injected with celecoxib had 15% mortality rate during the first few days of life (Table 1). One possible explanation for these findings is an essential role for COX-2 activity early in life that is distinct from COX-1. This interpretation is supported by the 55% neonatal mortality rate observed in the COX-2 transgenic mice (Table 2). This could be due

to the role COX-2 plays in closure of the ductus arteriosus, which is a critical event for directing deoxygenated blood from the heart into the lungs following birth [26]. Previous studies have reported that newborn COX-2 transgenic mice have difficulty closing the ductus arteriosus [25, 27, 28]. Later in the course of hyperoxia exposure there were no differences in mortality across treatments or genotypes.

Hyperoxic exposure increased BAL protein concentrations, a marker of lung injury. Neither aspirin nor celecoxib treatment decreased BAL protein concentrations in 85% O₂ -exposed pups (Figure 2). In contrast, 85% O₂ -exposed COX-2^{-/-} pups had reduced BAL protein concentration compared to 85% O₂ -exposed WT pups. These data suggest that COX-2 may regulate mechanisms that affect vascular and alveolar permeability and that pharmacological inhibition was not sufficient to cause biologically measurable changes. In other models of lung injury, COX-2 metabolites, including TXB₂ and PGE₂, have been implicated in regulating vascular permeability in the lung [29, 30].

Previous reports have identified the production of chemoattractant molecules and subsequent leukocyte infiltration as pivotal events in the course of newborn hyperoxic lung injury [31–34]. We used leukocyte chemoattractants, KC and MCP-1, as markers of leukocyte chemotaxis. Hyperoxia-exposure induced increases in both markers, however, administration of COX inhibitors did not attenuate MCP-1 or KC levels (Figure 3A). Further enhancement of KC levels in aspirin-treated, 85% O₂ -exposed pups is likely mediated via COX-independent mechanisms. Aspirin has been shown to have COX-independent effects including altering MAPK and NFκB-mediated signaling pathways [35]. Of interest, however, is our finding that 85% O₂ -exposed COX-2^{-/-} pups had substantially lower BAL KC and MCP-1 protein levels than did WT or COX-2^{+/-} pups (Figure 3B). The effects of COX-2 on KC and MCP-1 may be related to altered COX activity.

Inflammatory cell infiltration was further investigated by immunohistochemical staining for macrophages in lung tissue sections. Increases in the number of macrophages present in the lung parenchyma were evident due to hyperoxia exposure and these increases were attenuated by suppression of COX-2 activity (Figure 4A). We speculate that COX-2 activity may affect the expression of other macrophage chemoattractants which could have a role in newborn hyperoxic lung injury. Alternatively, aspirin and celecoxib treatment could have affected other molecules that regulate for macrophage diapedesis into the lung.

Since macrophage infiltration is attenuated in the absence of COX-2 activity [31], we hypothesized that alveolarization would be preserved in the treated or knockout pups. The hyperoxia-induced deficits in lung development, as assessed by alveolar number and area, were not associated with alterations in COX activity (Figure 5). We observed significant differences in alveolar number between 21% O₂ wild type and COX^{+/-} pups; however these differences were small and not likely to be biologically significant. More importantly, the decreases in inflammatory responses did not result in preservation of alveolarization. These findings suggest that alveolar development may not be directly impacted by inflammatory mediators and macrophage infiltration in the murine BPD model used in these studies.

The activity of COX can be quantified by assessing the production of its metabolites, prostaglandins (Figure 6). Numerous products including PGD₂, PGE₂ and TXB₂ were detected in lung tissues of 14 day old pups, however, measurement of other less well defined metabolites in the BAL were below limit of detection (data not shown). Our data indicate that aspirin treatment and to a lesser extent celecoxib treatment attenuated hyperoxia-induced production of prostaglandins, particularly those within the PGD₂ pathway (Figure 7A). In contrast to celecoxib-injected and COX-2^{-/-} pups, aspirin treatment also affected PG levels in 21% O₂-exposed pups (Figure 7). Despite no effect in 21% O₂ exposed pups, celecoxib inhibited hyperoxia-induced increases in PGE₂ and 13, 14-dihydro-15-keto-PGD₂ (Figure 7A). Aspirin and celecoxib differ in their selectivity for COX-1 and COX-2 [35]. Based on our data, we speculate that COX-1 activity may be a critical contributor to constitutive prostaglandin production in the lung during 21% O₂ exposure and its activity would not be altered by celecoxib, while COX-2 may contribute to the hyperoxia-induced increases in prostaglandin levels.

No differences in PG production were observed in the transgenic heterozygous or homozygous mice compared to WT mice in 21% O₂ (Figure 7B). However, there were trends towards decreased PGD₂ and TXB₂ levels in COX-2^{-/-} pups exposed to hyperoxia compared to WT mice exposed to hyperoxia (Figure 7B). Compensatory activity of COX-1 in response to loss of COX-2 activity may be responsible for the lack of differences between WT and transgenic mice exposed to hyperoxia. Interestingly, there were differences in lung concentrations of PGD₂ and its respective metabolites between C57Bl/6 and C3H/HeN mice (Figure 7). This may be due to the differences in genetic background and/or activity of secondary enzymes, such as HPGDS, that are responsible for metabolism of PGH₂ into bioactive prostaglandins.

PGD₂ is enzymatically metabolized by 15-hydroxy-prostaglandin dehydrogenase to form 13, 14-dihydro-15-keto-PGD₂ [36]. Recent studies have suggested that PGD₂, 13, 14-dihydro-15-keto-PGD₂, and PGE₂ regulate macrophage chemotaxis [37–40]. Prostaglandins have been shown to influence macrophage activation and their ability to produce chemokines [41, 42]. Our data suggest that macrophage infiltration during hyperoxia exposure could, at least in part, be due to increases in expression of PGD₂ metabolites and PGE₂.

Expression of another PGD₂ metabolite, 15-deoxy- 12, 14-PGJ₂, was reduced by aspirin and celecoxib treatment during exposure to hyperoxia (Figure 7). 15-deoxy- 12, 14-PGJ₂ has been shown to have anti-inflammatory effects in rodent models of lung injury [43, 44]. However, 15-deoxy- 12, 14-PGJ₂ has also been shown to stimulate lymphocyte chemotaxis [39]. Our data suggest that 15-deoxy- 12, 14-PGJ₂ may have pro-inflammatory effects in the lung during exposure to hyperoxia. Since suppression of 15-deoxy- 12, 14-PGJ₂ levels was associated with reduced macrophage infiltration, we speculate that reduction in 15-deoxy- 12, 14-PGJ₂ levels may also play a role in reduced macrophage infiltration into the lung in our model.

Aspirin and celecoxib also inhibited hyperoxia-induced PGE₂ levels. PGE₂ has been suggested to impact pro-inflammatory and inflammatory resolution responses in the lung

[20, 45]. Recent studies suggest that PGE₂ regulates pulmonary edema and immune cells during lung inflammation injury [29, 46]. Increased levels of PGE₂ have also been shown to influence “class switching” which is thought to be a pivotal event for the transition into inflammatory resolution pathways [47].

Our findings indicated that there was an increase in prostaglandin D and E synthase activity in C3H/HeN pups. We found that protein expression of prostaglandin synthases, HPGDS and MPGES, were significantly increased in lungs from hyperoxia-exposed pups (Figure 8A and 8B). Additionally, we detected expression of HPGDS in airway epithelial cells and MPGES in cells in alveolar walls and airspaces (Figure 8C). We speculate that the airway epithelium releases PGD₂, while the alveolar epithelium contributes to PGE₂ levels in the lung. Additional studies will be needed to interrogate the specific effects of PGD₂ and PGE₂ and their cell specific expression during newborn hyperoxic lung injury.

The role of COX-2 during inflammatory events in the lung is complex. Studies have suggested that COX-2 regulates pro-inflammatory and anti-inflammatory responses [20, 48]. Furthermore, the effects of COX-2 activity are impacted by the actions of prostaglandins that are produced. We found that inhibition of COX-2 reduced macrophages and altered prostaglandin levels in the lung. COX activity may influence pro-inflammatory responses, particularly leukocyte infiltration, in preterm infants that receive supplemental oxygen. Based on our data, we speculate that altering COX-2 activity influences hyperoxia-induced inflammation but does not positively affect lung alveolarization. Although COX-2 inhibition did not lead to protection against hyperoxia-induced alveolar deficits, COX-2 could be a useful therapeutic target to reduce inflammation in the lung of preterm infants at risk of developing BPD.

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Abbreviations

15-hydroxy-PGD	15-hydroxy-prostaglandin dehydrogenase
BAL	bronchoalveolar lavage
BPD	bronchopulmonary dysplasia
COX	cyclooxygenase
HPGDS	hematopoietic PGD synthase
IL	interleukin
KC	Keratinocyte-derived chemokine
MCP-1	monocyte chemoattractant protein-1
MPGES	microsomal PGE synthase

PG	prostaglandin
TX	thromboxane
TXAS	thromboxane A ₂ synthase

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Highlights

1. Hyperoxia exposure increased cyclooxygenase-2 expression and activity in the lung.
2. Cyclooxygenase-2 has a role in hyperoxia-induced lung inflammation in newborn mice.
3. COX inhibitors, aspirin and celecoxib, can modulate prostaglandin levels in the newborn mouse lung.

Figure 1.

COX-2 and COX-1 expression in the lung. Newborn C3H/HEN pups were exposed to 21% O₂ or 85% O₂ for 14 days. (A) COX-2 and (B) COX-1 protein levels were measured in lung homogenates by Western blot. Data were analyzed by unpaired student's t test. Data represents mean $\pm$ SEM, n=6 per group. Symbol indicates significant difference, p<0.05: (*) different than 21% O₂.

Figure 2.

Bronchoalveolar lavage fluid protein concentration expression. (A) Newborn C3H/HEN pups were injected daily with vehicle, 40 mg/kg aspirin, or 5 mg/kg celecoxib or (B) wild type and COX-2 transgenic mice were exposed to either 21% or 85% O₂ for 14 days. Protein concentration in BAL was measured by bradford assay. Data represents mean $\pm$ SEM, n=4–12 per group. Data were analyzed by two-way ANOVA followed by Newman-Keuls or Tukey's multiple comparisons test. Symbols indicate significant differences, p<0.05: (*) different than 21% O₂/vehicle; (#) different than 21% O₂/aspirin, (^)different than 21% O₂/celcoxib, (%) different than 21% O₂/COX^{-/-}.

Figure 3.

KC and MCP-1 levels in bronchoalveolar lavage fluid. (A) Newborn C3H/HEN pups were injected daily with vehicle, 40 mg/kg aspirin, or 5 mg/kg celecoxib or (B) wild type and COX-2 transgenic mice were exposed to either 21% O₂ or 85% O₂ for 14 days. KC and MCP-1 levels were measured in BAL by ELISA. Data represents mean $\pm$ SEM, n=4–12 per group. Data were analyzed by two-way ANOVA followed by Newman-Keuls or Tukey's multiple comparisons test. Symbols indicate significant differences, $p < 0.05$. Panel A: (*) different than 21% O₂/vehicle; (#) different than 21% O₂/aspirin; (^) different than 21% O₂/celecoxib; (\$) different than 85% O₂/vehicle. Panel B: (%) different than 21% O₂/COX-2^{+/+}; (§) different than 21% O₂/COX-2^{+/-}; (§) different than 21% O₂/COX-2^{-/-}.

Figure 4. Macrophages in lung tissues. (A) Newborn C3H/HEN pups were injected daily with vehicle, 40 mg/kg aspirin, or 5 mg/kg celecoxib or (B) wild type and COX-2 transgenic mice were exposed to either 21% O₂ or 85% O₂ for 14 days. Lung sections were immunohistochemically stained with an antibody specific for the macrophage surface marker, F4/80. Brown indicates positive staining for F4/80. Data represents mean $\pm$ SEM, n=2–4 per group. Data were analyzed by two-way ANOVA followed by Tukey's or Newman-Keuls multiple comparisons test. Symbol indicates significant difference, p<0.05: (*) different than 21% O₂/vehicle; (%) different than 21% O₂/COX^{+/+}.

Figure 5.

Alveolar development. (A) Newborn C3H/HEN pups were injected daily with vehicle, 40 mg/kg aspirin, or 5 mg/kg celecoxib or (B) wild type and COX-2 transgenic mice were exposed to 21% O₂ or 85% O₂ for 14 days. Lung sections were stained with H&E and alveolar numbers, areas, and perimeters were determined in five independent area per slide using digital analysis software. Data represents mean $\pm$ SEM, n=2–10 per group. Data were analyzed by two-way ANOVA followed by Newman-Keuls multiple comparisons test. Symbols indicate significant differences, $p < 0.05$: (*) different than 21% O₂/vehicle; (#) different than 21% O₂/aspirin; (^) different than 21% O₂/celecoxib; (%) different than 21% O₂/COX^{+/+}.

Figure 6.

Prostaglandin synthesis pathway. Arachidonic acid is released from membrane bound phospholipids by phospholipase A₂. COX-1 and COX-2 metabolize arachidonic acid into PGH₂. Specific synthases including hematopoietic prostaglandin D synthase (HPGDS), microsomal prostaglandin E synthase (MPGES), and thromboxane A₂ synthase (TXAS) metabolizes PGH₂ into PGD₂, PGE₂, and TXA₂, respectively. PGD₂ can be enzymatically converted into 13, 14-dihydro-15-keto-PGD₂ by 15-hydroxy-prostaglandin dehydrogenase (15- PGDH). Additionally, PGD₂ nonenzymatically converts, via dehydration, into PGJ₂ which upon additional dehydration converts into 15-deoxy- 12, 14-PGJ₂. Thromboxane A₂ synthase produces TXA₂ which nonezymatically converts into the stable metabolite, TXB₂.

Figure 7.

Prostanoid levels in lung tissues. Prostaglandin levels were measured in lung tissues by LC/MS-MS. (A) Newborn C3H/HEN pups were injected daily with vehicle, 40 mg/kg aspirin, or 5 mg/kg celecoxib or (B) wild type and COX-2 transgenic mice were exposed to 21% O₂ or 85% O₂ for 14 days. Prostanoid levels were measured LC-MS/MS using MRM and isotope dilution. Data represents mean ± SEM, n=2–7 per group. Data were analyzed by two-way ANOVA followed by Newman-Keuls or Tukey's multiple comparisons test. Symbols indicate significant differences, p<0.05. (*) different than 21% O₂/vehicle; (#) different than 21% O₂/aspirin; (\$) different than 85% O₂/vehicle; (^) different than 21% O₂/celecoxib (+) different than 85% O₂ celecoxib.

Figure 8.

Expression of prostanoid synthases in the lung. Newborn C3H/HEN pups were exposed to 21% O₂ or 85% O₂ for 14 days. (A) HPGDS and (B) MPGES protein levels in the lung were measured by Western blot. Data represents mean $\pm$ SEM, n=5–6 per group. Data were analyzed by two-way ANOVA followed by Newman-Keuls multiple comparisons test. Symbol indicates significant difference, $p < 0.05$: (*) different than 21% O₂. (C) Lung sections were stained with antibodies specific for HPGDS and PGES. Expression of HPGDS was identified in airway epithelial cells while expression of PGES was detected in alveolar walls and cells that could be macrophages. Positive cells are indicated by arrows.

Table 1

Body Weights and Survival of C3H/HEN mice.

	21% O₂ + Vehicle	21% O₂ + 40 mg/kg Aspirin	21% O₂ + 5 mg/kg Celecoxib	85% O₂ + Vehicle	85% O₂ + 40 mg/kg Aspirin	85% O₂ + 5 mg/kg Celecoxib
Body Weight (g)	8.32 ± 0.447	7.57 ± 0.114	8.42 ± 0.101	7.55 ± 0.253	7.41 ± 0.139	7.59 ± 0.141 ^(*)
Survival (%)	100	100	85.71	92.86	100	96
n	22	29	26	24	27	27

Significant effects of exposure and injection were observed. Data represents mean ± SEM, n=22–29. Data were analyzed by log-rank (Mantel-Cox) test or two-way ANOVA followed by Newman-Keuls multiple comparisons test.

^(*) 21% O₂/vehicle, p<0.05.

Table 2

Body Weights and Survival of wild type and COX-2 transgenic mice.

	21% O ₂ + COX-2 ^{+/+}	21% O ₂ + COX-2 ^{+/-}	21% O ₂ + COX-2 ^{-/-}	85% O ₂ + COX-2 ^{+/+}	85% O ₂ + COX-2 ^{+/-}	85% O ₂ + COX-2 ^{-/-}
Body Weight (g)	6.74 ± 0.225	6.07 ± 0.173	4.98 ± 0.926	5.64 ± 0.429	5.63 ± 0.265	4.53 ± 0.281 ^(*)
Survival (%)	100	89.27	43.48 ^(*)	100	95.65	47.06 ^(*)
n	16	28	23	20	23	17

Significant effects of genotype were observed. Data represents mean ± SEM, n=16–28. Data were analyzed by log-rank (Mantel-Cox) test or two-way ANOVA followed by Newman-Keuls multiple comparisons test.

^(*) 21% O₂/WT, p<0.05.