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Original Contributions

Cyclooxygenase-2 in newborn hyperoxic lung injury

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ABSTRACT

Supraphysiological O_2 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 micewere injected daily with vehicle, aspirin (nonselective 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.Micewere exposed to room air (21% O_2) or hyperoxia (85% O_2) 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 air spaces.Aspirin and celecoxib treatment attenuated hyperoxia-induced COX activity, including altered levels of prostaglandin (PG)D_2 metabolites.Decreased COX activity, however, did not prevent hyperoxia-induced lung developmental deficits.Our data suggest thatincreased COX-2 activity may contribute to proinflammatory 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.

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 O_2 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], contributes to the development of BPD [6,7]. Preterm infants at risk of developing BPD have increased expression of many proinflammatory mediators including interleukin (IL)-6, IL-8, IL-1β, and IL-10 [8].Multiple studies have reported increased levels of leukocyte and proinflammatory 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)H_2. Subsequently, PGH_2 becomes a substrate for synthases that metabolize PGH_2 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 PGD_2, PGE_2, and thromboxane (TX)B_2 have been shown to regulate

Abbreviations: 15-hydroxy-PGD, 15-hydroxy-prostaglandin dehydrogenase; BAL, bronchoalveolar lavage; BPD, bronchopulmonary dysplasia; COX, cyclooxygenase; HPGDS, hematopioetic 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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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 are 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 nonselective 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% O_2) or hyperoxia (85% O_2) for 14 days.Our findings suggest that COX-2 has a proinflammatory 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% O_2) while the corresponding litter was placed in hyperoxia (85% O_2) 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 21or 85% O_2 for 14 days.To avoid oxygen toxicity, nursing dams were rotated daily between the 21%and th 85% O_2 paired litters every 24 h.After 14 daysof 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 3x with sterile PBS.Lavage fluid was centrifuged at 3000 rpm for 10 min 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), hematopioetic 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 h.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 H_2O and embedded in paraffin.To assess alveolarization, lung sections were stained with H&E and five nonoverlapping, 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), hematopioetic 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 the manufacturer's 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 NaH_2PO_4, 0.9% NaCl buffer at pH 5.An internal standard containing 0.5 ng/μL each of deuterated $PGF_{2\alpha}$, TXB_2, PGD_2, leukotriene B_4, 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 N_2. 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 Tukeys or 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 expressionin lung tissues

Pulmonary COX-2 and COX-1 protein expression was measured in lung homogenates obtained from 21 or 85% O_2-exposed pups by