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Respiratory Syncytial Virus (RSV) Infection Induces Cyclooxygenase 2: A Potential Target for RSV Therapy

Joann Y. Richardson,* Martin G. Ottolini,* Lioubov Pletneva,§ Marina Boukhalova,§ Shuling Zhang,† Stefanie N. Vogel,‡ Gregory A. Prince,§ and Jorge C. G. Blanco2§

Cyclooxygenases (COXs) are rate-limiting enzymes that initiate the conversion of arachidonic acid to prostanooids. COX-2 is the inducible isof orm that is up-regulated by proinflammatory agents, initiating many prostanoid-mediated pathological aspects of inflammation. The roles of cyclooxygenases and their products, PGs, have not been evaluated during respiratory syncytial virus (RSV) infection. In this study we demonstrate that COX-2 is induced by RSV infection of human lung alveolar epithelial cells with the concomitant production of PGs. COX-2 induction was dependent on the dose of virus and the time postinfection. PG production was inhibited preferentially by NS-398, a COX-2-specific inhibitor, and indomethacin, a pan-COX inhibitor, but not by SC-560, a COX-1-specific inhibitor. In vivo, COX-2 mRNA expression and protein production were strongly induced in the lungs and cells derived from bronchoalveolar lavage of cotton rats infected with RSV. The pattern of COX-2 expression in vivo in lungs is cyclical, with a final peak on day 5 that correlates with maximal histopathology. Treatment of cotton rats with indomethacin significantly mitigated lung histopathology produced by RSV. The studies described in this study provide the first evidence that COX-2 is a potential therapeutic target in RSV-induced disease. The Journal of Immunology, 2005, 174: 4356–4364.

Respiratory syncytial virus (RSV)1 is the leading viral cause of death in children under 1 year of age and is an increasing cause of morbidity and mortality in transplant patients and the elderly (1–3). RSV causes upper and lower respiratory tract infections, occasionally leading to severe bronchiolitis and pneumonia. In addition, RSV bronchiolitis has been associated with the development of recurrent episodes of bronchiolar obstruction, specific IgE production, and establishment of asthma (4–6). It is unclear why children, the elderly, and the immunosuppressed are at much higher risk for severe disease; however, an RSV-induced immune pathological mechanism has long been suspected. Yet there is no safe and effective vaccine against RSV. Passive anti-RSV Ab, although effective in prophylactic settings, does not provide any clinically beneficial outcome when applied therapeutically, indicating that RSV-induced pathology is primarily the result of the inflammatory response to infection, rather than a direct viral effect. Therefore, a combined antiviral and anti-inflammatory therapy might represent the most safe and efficient treatment against RSV infection.

During RSV infection, proinflammatory cytokines and chemokines are detectable in isolates from bronchoalveolar lavage (BAL) fluids of patients with lower respiratory tract infection (7–10). Many inflammatory mediators induced in the lung by RSV are effective inducers of the cyclooxygenase 2 (COX-2) in lung epithelial and inflammatory cells (11–15). The expressions of COX-2 and its products, PGs and thromboxanes (TBx), have been correlated with the development of many inflammatory processes (16, 17), some of which occur during viral infection (e.g., regulatory effects on the immune response, vascular tone, platelets aggregation, airway remodeling, allergic processes, etc.). Moreover, RSV induces the production of PGE2 in cultures of human monocytes and dendritic cells (18). The potent physiologic effects of PGs and TBx and their possible role in RSV infection suggest that these mediators should be considered as potential important factors in the RSV disease process. The animal model of choice for RSV studies is the cotton rat (Sigmodon hispidus), because it most closely resembles disease in humans (19). Preclinical data from cotton rats have resulted in a successful prophylactic approach in humans (20–24).

In this work we present data demonstrating the induction of COX-2 expression during RSV infection in vitro, in human lung alveolar epithelial cells and cotton rat macrophages, and in vivo, in the lungs of cotton rats infected with RSV. We present additional evidence that indicates the potential of nonsteroidal anti-inflammatory drugs in the treatment of RSV-induced bronchiolitis.

Materials and Methods

Animals

Inbred cotton rats (S. hispidus) were obtained from a colony maintained at Virion Systems. Cotton rats were housed in large polycarbonate rat cages with a bedding of pine shavings (Harlan Teklad) and were fed a diet of rodent chow and water. The cotton rat colony was monitored for Abs to paramyxoviruses, RSV, and rodent viruses; no such Abs were found. The animals used for infection experiments were, on the average, 8–12 wk old and weighed 100 g at the time they were used. All animal experimentation procedures were performed following National Institutes of Health and U.S. Department of Agriculture guidelines with institutional animal care and use committee approval.

Virus and tissue culture cells

The Long strain (group A) of RSV was obtained from American Type Culture Collection. Virus stocks were prepared in HEp-2 cells and contained 1 × 10^7.5 PFU/ml. Viral titers in stocks and lung homogenates were
determined by plaque assay on HEp-2 cells (19). A549 cells were obtained from American Type Culture Collection and were grown in DMEM supplemented with 2 mM l-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FBS in a humidified 37°C incubator with 5% CO2. For RSV infection, cells were seeded in a six-well plate at a density of 4 x 10^6 cells/well 1 day before infection. The cells were generally 70–80% confluent on the day after seeding. Purified stocks of RSV were diluted in DMEM to yield multiplicities of infection (m.o.i.) of 1, 0.5, and 0.1. The media were removed from the cells and replaced with medium containing virus dilutions for 1 h, then replaced with complete DMEM. Uninfected HEp-2 cell culture supernatant served as the control (mock inoculum). Cells were harvested at various times postinfection, and the supernatants were stored at −70°C for quantification of PGE2, by ELISA (Cayman Chemicals). For inhibition experiments of COX enzymes in A549 cells, infected cells were incubated with different concentrations of indomethacin (in saline), Indocin, a pan-COX inhibitor (Merck); NS-398 (in DMSO), a COX-2-specific inhibitor (Cayman Chemicals); SC-560 (in DMSO), a COX-1-specific inhibitor (Cayman Chemicals); or the vehicles as controls. For analysis of the supernatants of infected A549 cells for the presence of soluble factors that cause COX-2 activation, the supernatants were filtered through Centricon columns (Centricon Centrifugal Filter Devices, YM-10, 100,000 kDa cutoff; catalogue no. 4212; Amicon Bioseparations) and incubated with 50 μg/ml of the following Abs: anti-human IL-1α (R&D Systems; catalogue no. MAB200), anti-human IL-1β (R&D Systems; catalogue no. MAB201), anti-human TNF-α (R&D Systems; catalogue no. MAB210), or control mouse IgG before adding them to the cells. Recombinant human (rh) IL-1α, IL-1β, rhTNF-α (1 ng/ml; BioSource International; catalogue no. PHC0314, PHC0814, and PHC3015, respectively) were used as positive controls in the indicated concentrations.

Peritoneal macrophages were prepared from cotton rats by injecting 6–8 ml of thioglycolate broth i.p. into each of 8–16 wk-old S. hispidus. Four days postinjection, rats were killed by CO2 inhalation, and the peritoneal cavity was washed twice with 30 ml of cold saline solution. Peritoneal exudates (~2 x 10^7 cells) were subjected to Western blot analysis using anti-COX-2 Ab as described previously (26).

Cloning of cotton rat cox-2 gene

A fragment encoding part of the cotton rat cox-2 coding sequence (1540 bp long) was amplified using cDNA obtained from LPS-activated cotton rat macrophages and the following primers obtained from comparison of human, mouse, and rat sequences: forward, 5'-CAGGAAATCTCTGTGTTTCTGCTGTTCCMYCCATGYYCARAAAGYWWGKGTATGTAGYGRYRGAGTTYGACCAR-3'; and reverse, 5'-GTATGGGAGGACGATTGGTTGGATGACCAC-3'. Amplification was performed using standard PCR reagents and cycle conditions. PCR-amplified products were subjected to agarose gel electrophoresis, and a single band of the predicted m.w. was isolated from the gel by QIAEX II gel extraction kit (Qiagen), quantified by UV spectrophotometry, sequenced, and labeled using the Dig DNA Labeling and Detection Kit (Roche). A cotton rat cDNA library (derived from LPS-activated peritoneal macrophages) was constructed using the plasmid vector pSPORT 1 (Invitrogen Life Technologies) and transferred into MAX Efficiency DH5α cells (Invitrogen Life Technologies). A total of 5000 colonies were screened, positive clones were isolated, and a full-length clone encoding the cotton rat cox-2 gene was entirely sequenced.

RT-PCR analysis

Semi-quantitative analysis of COX-2 mRNA expression in lungs of RSV-infected cotton rats by RT-PCR and Southern blot analysis was performed similarly to that previously described for other cotton rat genes (27). The following primers and conditions were used: forward primer, 5'-CATT GACGAGCCAGGAGTAGAATAC-3'; reverse primer, 5'-GTACTGTTGGGATATCATCAGTGC-3'; and probe, 5'-AAGGCTTATGAATGATGATGAC-3', using 22 amplification cycles and 65°C for the annealing temperature. For analysis of viral gene expression, the RSV F protein gene was amplified using the following primers: forward primer, AACCTCTAAAGCAATGGCATTAC; and reverse primer, ATGGCCTCCTAGAGATGTGATAACGGAGC, using 32 (macrophages) or 25 (A549 cells) amplification cycles and 55°C for the annealing temperature. The product of the reaction (a 1.2-kb band) was visualized by direct ethidium bromide staining of agarose gel after electrophoresis.

COX-2 Western blot and immunoprecipitation

A549 cells were homogenized in lysis buffer containing 20 mM Tris-HCl (pH 8), 100 mM NaCl, 1% Nonidet P-40, 4 mM DTT, 0.5 mM PMSF, 7.5 mM sodium fluoride, 2 mM EDTA, and a mixture of protease inhibitors (Complete; Roche). Cell lysates were normalized by protein concentration, and 40 μg/lane was subjected to electrophoresis in 10% SDS-PAGE gels. Western blot analysis was used to detect COX-2 protein using a polyclonal rabbit Ab (catalogue no. 160116; Cayman Chemicals) directed against the epitope located between aa 584 and 598 (HSHSLDDNPVTLLK) of the mouse COX-2 protein, which is 100% conserved in the cotton rat COX-2, and detected via secondary HRP-conjugated anti-rabbit Abs (made in the donkey; Amersham Biosciences; catalogue no. NA934) and the ECL detection system (Amersham Biosciences).

For detection of COX-2 protein in the lungs of RSV-infected cotton rats, the left lung of each rat was homogenized in 2 ml of lysis buffer using a microhomogenizer ( Omni International). The homogenates were centrifuged at 4000 x g for 15 min at 4°C to remove cell debris. Each supernatant was sonicated (10 s with microtip at 4°C) and finally centrifuged at 16000 x g for 10 min. Four hundred microliters of each lung homogenate was incubated with 800 ng of anti-COX-2 Ab (sc-1746; Santa Cruz Biotechnology) and mixed by rotation (30 rpm) for 3 h at 4°C. Immunocomplexes were precipitated with ImmunePure-immobilized protein G-Sepharose (50% slurry in lysis buffer; Pierce) and washed three times with lysis buffer, and the pellet was electroeluted and analyzed by Western blot for COX-2. β-Actin Abs (sc-8432; Santa Cruz Biotechnology) were used to normalize the amount of protein extract used for immunoprecipitation.

Animal protocol

Cotton rats were inoculated intranasally under isoflurane (Abbott Laboratories) anesthesia with 100 μl of RSV/Long suspension containing 1 x 10^8 50% PFU. Controls included uninfected cotton rats and cotton rats inoculated intranasally with mock inoculum. Animals were killed, a midline vertical chest incision was made to expose the thoracic contents, and the lungs were removed en bloc. The right lung was ligated and submitted for viral titration (19). BAL was performed by inserting a blunt 20-gauge needle into the trachea, which was secured in place by ligature. Three milliliters of cold PBS was introduced, and the pulmonary tree was laved. The recovered sample was centrifuged at 800 x g, and the cell pellet was resuspended in lysis buffer after counting and volume normalization. Cell lysates were stored at −70°C until analyzed. Intrapulmonary injection of indomethacin (Indocin; Merck; 0.3 mg/kg in saline solution) was administered 12 h before inoculation with RSV/Long and subsequently at 12 and 36 h postinfection. Control groups consisted of uninfected animals, mock-infected animals, and RSV-infected, sham-treated animals (i.p. injection of saline). Animals were killed on day 4 postinfection, and lungs were removed en bloc for viral titration and histopathology.

Inhibition of COX-2 activity in an ex vivo-stimulated whole blood assay

Blood (~1 ml) was collected by cardiac puncture in heparinized tubes for TXB2 analysis from animals treated i.p. with indomethacin (0.3 mg/kg) or saline three times at 24-h intervals. To determine the extent to which indo- methacin inhibited COX activity in vivo, TxB2 (a subproduct of TxA2) was measured from whole blood platelets after stimulation with a calcium ionophore (A23187) as previously described (28).

Histopathology

Formalin-fixed lung tissues were embedded in paraffin, and 4-μm sections were obtained and stained with H&E (Histology). One H&E-stained slide was prepared per animal killed. Three histopathologic parameters were examined on each H&E-stained slide: peribronchiolitis, alveolitis, and interstitial pneumonitis. Peribronchiolitis is defined as inflammatory cells, mostly lymphocytes and macrophages, that accumulate around the periphery of small airways. Alveolitis is defined as inflammatory cells, mostly macrophages and neutrophils, within the airspace. Interstitial pneumonitis is defined as thickening of the alveolar wall, associated with an influx of inflammatory cells of various types. Each parameter was scored separately
under blinded conditions and was assigned a score based on a scale of 0 (no inflammation) to 4 (maximum inflammation) as previously described (29).

Statistics

Arithmetic means were calculated for each of the three histopathologic parameters obtained for each group of cotton rats. The RSV-infected/indomethacin treated group was compared with the RSV-infected/untreated group using the two-tailed Student’s t test.

Results

Induction of COX-2 expression and PGE\textsubscript{2} production by RSV in vitro

The human type II pneumocyte cell line, A549, has been used extensively in RSV studies in vitro. A549 cells are highly permissive to RSV infection and produce large syncytia and high levels of cytokines and chemokines, such as IL-1 (30), TNF-\textalpha (30), IL-6 (31), and IL-8 (31, 32). To investigate whether COX-2 was also inducible by RSV in these cells, A549 cell monolayers were infected with RSV at various m.o.i. and harvested at different times postinfection.

Infected cell lysates were evaluated for the presence of COX-2 and viral mRNA by RT-PCR (Fig. 1A) and for protein by Western analysis (Fig. 1B). Induction of steady-state levels of COX-2 mRNA was detected in cells infected with higher m.o.i. (1 and 0.5) by 40 h postinfection. When an m.o.i. of 0.1 was used, a significant increase in the level of COX-2 mRNA expression was seen only after 44 h. At 48 h, the steady state levels of COX-2 mRNA peaked at all m.o.i. of virus (Fig. 1A, graph), and the expression of \beta-actin, a housekeeping gene, was slightly reduced, consistent with the observed cytopathic effect of the virus on the cell monolayer. The steady state levels of expression of the gene that encodes the RSV F protein were constant throughout the period monitored in infected cells. COX-2 protein expression was undetectable during the first day of infection, but peaked during the second day postinfection, with the highest m.o.i. giving the strongest COX-2 signal (Fig. 1B), consistent with the mRNA expression data.

Induction of COX-2 protein was also dependent on viral replication in these cells, because treatment of virus with UV light, or palivizumab, an anti-RSV F protein mAb (Synagis; MedImmune) did not lead to COX-2 mRNA (Fig. 2A) and protein expression (Fig. 2B). At the time, the expression of RSV F protein mRNA was undetectable, and no replicative virus was observed. Together, these data demonstrate that COX-2 is induced during RSV infection in human lung epithelial cells and requires infection with replicative viral particles.

Because mRNA expression of COX-2 in these cells was detected at \textasciitilde40 h postinfection, its induction is more likely to be secondary to the action of a secreted factor(s) induced by viral infection in these cells. To test this hypothesis, we incubated A549 cells for only 6 h with several dilutions of filtered (virus-free) supernatants obtained from cultures of A549 cells that were infected for 48 h with different m.o.i. of RSV (Fig. 3A). In contrast to undiluted control supernatants, these supernatants from virus-infected cells strongly activated the expression of COX-2 in a dose-dependent manner. IL-1 (33, 34) and TNF (14) are known to be potent inducers of COX-2. They are secreted in A549 cells after RSV infection (30). We used blocking Abs against IL-1\textalpha, IL-1\beta, and TNF-\textalpha to determine whether these cytokines contribute to COX-2 mRNA expression when released into the supernatants by RSV-infected cells. Incubation of culture supernatants of RSV-infected A549 cells with Abs against these cytokines produced a marked reduction of COX-2 mRNA expression (Fig. 3B). Blocking of IL-1\textalpha had the strongest inhibitory effect on the activation of COX-2 mRNA expression by supernatants (compare lane 1 with lane 10), whereas blocking TNF-\textalpha and IL-1\beta partially inhibited COX-2 mRNA expression (compare lanes 4 and 7 with lane 10). Taken collectively, these data demonstrate that COX-2 mRNA and protein expression is strongly induced in human lung epithelial cells by RSV. The induction is dependent on viral replication and requires the action of soluble factors, including IL-1\textalpha, IL-1\beta, and TNF-\textalpha, that act on the cells to stimulate COX-2 expression.

To determine whether COX-2 expression was associated with PG production in A549 cells, supernatants of cells infected with RSV were assayed for the presence of PGE\textsubscript{2}. As depicted in Fig. 4A, PGE\textsubscript{2} was not detected on day 1, but was detected 2 days postinfection with levels that correlated well with the expressed COX-2 mRNA and protein seen by Western blot (Fig. 1). PGE\textsubscript{2} levels reached maximum levels on day 3 for all m.o.i., when the cell monolayer was almost completely destroyed by the infection.

Treatment of A549 cells with the selective COX-2 inhibitor NS-398 (>50-fold selective for COX-2 over COX-1) (35) or indomethacin (a pan-COX inhibitor) resulted in stronger inhibition of COX-2 mRNA expression (compare lanes 4 and 7 with lane 10).
independent experiments. 

PGE2 synthesis than SC-560, a highly selective COX-1 inhibitor (28) (Fig. 4B), confirming that the PGE2 produced was the result of the enzymatic activity of COX-2, not COX-1. None of the inhibitors significantly affected RSV replication even when used at concentrations as high as 100 nM (data not shown). Moreover, PGE2 production was dependent on virus replication because it was not detected when the virus used for infection was UV-inactivated or preincubated with palivizumab (data not shown).

Cloning and characterization of cotton rat COX-2

The cotton rat is a powerful model for studying RSV infection in vivo. To analyze the pattern of gene expression of several pro- and anti-inflammatory mediators during RSV infection, we developed cotton rat reagents (27) that have been used to demonstrate that RSV infection induced the expression of a number of important cytokines and chemokines in the lungs of primary infected and reinfected cotton rats. Our present interest in COX-2 led us to clone the cotton rat COX-2 gene. Cotton rat COX-2 cDNA (GenBank accession no. AY065644) is 90.5, 90.3, and 85.4% identical with the rat (accession no. S67722), mouse (accession no. NM_011198), and human (accession no. L15326) homologues, respectively, with corresponding amino acid sequence homologies of 94, 93, and 87%.

LPS is one of the most powerful inducers of COX-2 in macrophages (26, 36, 37). To determine whether COX-2 expression is induced and can be detected in cotton rat cells, peritoneal exudate macrophages were treated with LPS (Fig. 5A, left panel; Fig. 5B, LPS lanes). A strong induction of a protein that migrated at the expected Mr of COX-2 protein (72 kDa) was detected with an Ab that recognizes the conserved C-terminal amino acid sequence of the COX-2 protein.

Previous studies demonstrated the infection of peritoneal macrophages and neonatal monocytes with RSV in vitro, which resulted in the expression of several cytokines, including IL-1, TNF-α, and IL-6 (38, 39). Moreover, Bartz et al. (18) showed that PGE2 is produced by RSV-infected human macrophages and dendritic cells. To investigate whether COX-2 is induced in cotton rat macrophages during RSV infection, purified peritoneal macrophages and bronchioalveolar macrophages were isolated from cotton rats and infected with RSV at m.o.i. of 0.5 and 1, as indicated (Fig. 5). We found an increase in the expression of COX-2 protein 1 day postinfection, which persisted on day 2 with levels of COX-2 protein that approach those obtained after stimulation of macrophages with LPS. Virus replicates very inefficiently in these cells, with only marginal levels of infective virus obtained 2 days postinfection (between 2 and 3 log10 less that what was seen in A549 cells), but the expression of the RSV F protein gene was detectable by RT-PCR. Taken together, these data indicate that both lung epithelial cells and macrophages (peritoneal and bronchioalveolar) are potential sources of COX-2 and PG during infection with RSV.

RSV induction of COX-2 in vivo

To investigate in vivo the expression of COX-2 during RSV infection, cotton rats were infected intranasally with 106.5 PFU. On different days after infection, animals were killed, and total lung RNA was isolated. Steady state levels of COX-2 mRNA were analyzed by RT-PCR (Fig. 6A). COX-2 mRNA expression was detected at very low levels in uninfected animals, whereas a small
increase was seen in animals inoculated with a mock preparation of the virus. However, the presence of RSV in the inoculum generated a triphasic pattern of COX-2 gene expression. In the first 6 h, the expression peaked at its maximum (>8-fold compared with uninfected animals and ~5-fold compared with mock-infected controls) and then strikingly diminished at 12 h postinfection. COX-2 mRNA levels slowly climbed to a second peak on day 1, again declining on day 2, and finally climbing again to a third peak on day 5. Interestingly, levels of β-actin mRNA expression were reduced on days 1 and 2, just before the viral titers in lungs reached maximum levels (data not shown). COX-2 mRNA slowly decreased after day 5, and on days 7 and 10 postinfection, its levels were indistinguishable from control values. These data indicate that RSV induces the expression of COX-2 mRNA in lungs of infected cotton rats.

To determine whether the change in steady state levels of COX-2 mRNA correlates with a change in the expression of COX-2 protein in the lung, we analyzed lung homogenates of RSV-infected cotton rats. Groups of animals were infected and killed at different times postinfection. At 12 h and 1 day postinfection, the samples taken from all animals tested demonstrated a strong induction of COX-2 protein. Cells in BAL of healthy (uninfected) animals were mostly macrophages (68 ± 6.27%; n = 10) with some granulocytes (~30%) and almost no lymphocytes. The number of granulocytes in the lung increased during the first day of RSV infection, whereas the number of lymphocytes increased starting on day 3 post-RSV infection (40). The expression of COX-2 persisted in some animals until day 5 postinfection (Fig. 6C), although levels were barely detectable. Taken together, these data clearly demonstrate the induction of COX-2 mRNA and protein expression in the lungs and cells of the BAL after RSV infection of cotton rats.

Effect of inhibition of COX activity on RSV-induced lung histopathology

To determine the role of COX in RSV pathogenesis, the effect of indomethacin treatment on RSV-induced lung pathology was evaluated. Cotton rats were treated with indomethacin i.p. at a dose 20 µg/100 g rat given 12 h before inoculation with RSV. This dose, administered i.v., is equivalent to that used to promote closure of the ductus arteriosus in infants. Indomethacin was readministered 2% of the lung tissue (19), Western analysis of whole lung homogenates was not sensitive enough to detect local mediators of inflammation. To increase the signal-to-noise ratio, lung homogenates were analyzed by Western blot for COX-2 and β-actin protein levels. Macrophages stimulated with 100 ng/ml LPS (LPS lanes) were included as a positive control. For negative controls (–), mock-treated macrophages were used. Detection of RSV F protein gene expression was assessed by RT-PCR and ethidium bromide staining of agarose gels.
Induction of COX-2 expression has been demonstrated for a handful of other viruses, including human CMV, human herpesvirus, EBV, encephalomyocarditis, and Theiler’s virus (42–46). In an in vivo study of bronchial biopsies from human volunteers experimentally infected with human rhinovirus, the levels of COX-2 expression and in vivo, in whole lung and BAL cells of cotton rats infected with RSV. To our knowledge, this is the first report that analyzes the expression of COX-2 during RSV infection.

Discussion

In the present study we have demonstrated that RSV infection results in induction of COX-2 gene and protein expression both in vitro, in human lung epithelial cells and cotton rat macrophages, and in vivo, in whole lung and BAL cells of cotton rats infected with RSV. To our knowledge, this is the first report that analyzes the expression of COX-2 during RSV infection.

Each slide: peribronchiolitis, alveolitis, and interstitial pneumonitis. Fig. 7A illustrates mean histopathology scores. Inhibition of cyclooxygenase activity by indomethacin resulted in a significant reduction in all three histopathological score parameters measured when compared with untreated or to mock-treated control groups (Fig. 7C shows representative views of lung histology from each group). There were no statistical differences in viral replication measured in the lungs of infected animals treated with vehicle or indomethacin on day 4 postinfection (10^4.6 ± 0.09 vs 10^4.5 ± 0.15 PFU/g), indicating that indomethacin treatment did not affect viral replication in vivo. The same regimen of indomethacin was tested in a separate group of animal to determine its bioavailability. Indomethacin treatment diminished TxB2 production (a nonenzymatic metabolite of TxA2) from whole blood platelets stimulated with calcium ionophore (A23187) by ~40% (Fig. 7B) compared with animals treated i.p. with saline. These data indicate that inhibition of COX activity by indomethacin is more likely the cause of the reduction in pathology scores observed in infected and treated animals.

FIGURE 6. RSV induction of COX-2 in vivo. Cotton rats were inoculated intranasally with 100 μl of RSV suspension containing 1 × 10^6.5 PFU. Animals were killed at the indicated intervals. Both uninfected (−) cotton rats and cotton rats inoculated with uninfected tissue culture supernatant (MOCK) were used as controls. A, RT-PCR densitometry for quantification of steady state levels of COX-2 mRNA normalized to β-actin mRNA. Determinations were made in triplicate. Each bar represents data collected from five or more rats, and the results shown are representative of three independent experiments. B, Western blot analysis of COX-2 protein levels in whole lung homogenates after immunoprecipitation with an anti-COX-2-specific Ab (Santa Cruz Biotechnology; sc-1746). Western blot was performed using a different anti-COX-2 Ab (Cayman Chemical; catalog no. 160116). Western blot for β-actin was performed as a control for protein loading consistency. The m and M lanes represent cotton rat macrophage lysates precipitated with a control normal rabbit sera or polyclonal rabbit anti-COX-2, respectively. C, COX-2 expression in BAL of RSV-infected cotton rats. Total cell extracts of BAL were immunoprecipitated and analyzed by Western blot for COX-2 as described in B. A Western blot for β-actin was performed as a control for protein loading consistency.

FIGURE 7. Inhibition of COX activity decreases RSV-induced bronchitis, alveolitis, and interstitial pneumonitis in cotton rats. A, Histopathology scores of bronchitis, interstitial pneumonia, and alveolitis. Each bar represents the arithmetic mean of eight to 10 animals ± SEM. *, Values in indomethacin-treated animals that are significantly decreased (p < 0.05) from those in infected, saline-treated groups. B, Groups of animals were treated with indomethacin (indo) or saline solution (saline) i.p. three times at 24-h intervals for each treatment reproducing a similar regimen as that in A. No virus infection was performed in these animals. Blood samples were collected 24 h after the last treatment and induced with the calcium ionophore (A23187) in vitro, and TxB2 production was measured by ELISA. *, TxB2 production was significantly decreased by indomethacin treatment (p < 0.05). C, Photomicrograph of H&E-stained lung tissue (×64). Normal lung architecture is seen in panel a (uninfected cotton rat). In panel b, lungs of RSV-infected cotton rats killed on day 4 postinfection are shown. Significant alveolar infiltration with inflammatory cells, peri-bronchiolar thickening with lymphocytic infiltration, and coarse interstitial thickening are seen. Panel c, RSV-infected cotton rats were treated i.p. with three doses of indomethacin. Significant reductions in alveolitis, bronchiolitis, and interstitial pneumonitis are seen.
COX-2 and PGE₂ release. These mediators are induced in A549 cells infected with RSV AND COX-2. We have demonstrated that the activity of these supernatants was consistent with virus-induced cytokines in an autocrine loop. First, we suggested that COX-2 induction in A549 cells was more likely the result of viral replication, acting directly or through combined action with TLR4 (57–59). Moreover, TLR4-deficient mice exhibit an impaired capacity to clear RSV from the lungs (57, 59). In addition, early activation of NF-κB in the lungs of infected mice was shown to be both alveolar macrophage- and TLR4-dependent (59). This initial interaction between the pathogen and host would be predicted to elicit a cascade of inflammatory mediators that, in turn, would potentiate the inflammation response associated with RSV. Taken together, these data suggest that a different mechanism of induction of COX-2 is used by macrophages (or dendritic cells) and lung epithelial cells; in macrophages, COX-2 induction is dependent upon TLR4 engagement and is independent of viral replication, whereas in epithelial cells, COX-2 induction requires viral replication.

Our results indicate that COX-2 mRNA and protein expression showed three peaks in lungs of cotton rats during primary infection with RSV (Fig. 6). The first was detected as soon as 6 h postinfection and coincided with the induction of mRNA for cytokines such as IL-1β and TNF-α and chemokines such as IFN-inducible protein-10 and GRO (M. Boukhvalova and J. C. G. Blanco, unpublished observations). Although we cannot rule out a possible role of these factors in the induction of COX-2 in vivo, their coexpression at this early time point most likely indicates the use of shared mechanisms of gene induction triggered during innate responses to the virus, rather than an induction through secondary mediators. The second peak on days 1 and 2 and the third peak on day 5 correlate with viral replication and pathology scores, respectively (29). Although the final eicosanoid products that are synthesized during each of these instances are potentially different and may have different impacts on lung physiology, our data indicate that partial inhibition of eicosanoid production during infection is beneficial for the pathological outcome. Eicosanoid function in the airways has been subdivided into stimulatory PGs (bronchodilators) such as PGD₂, PGE₂α, and TBXs, on the one hand, and inhibitory PGs (bronchodilators) such as PGE₂, on the other (60). PGE₂ is the most abundant PG in lungs (60) and is considered to be a potent immunosuppressor. In vitro, PGE₂ activates the Th2 subset of T cells while suppressing the Th1 subset (61). PGE₂ suppression of Th1 responses is due to the selective and dose-dependent inhibition of IFN-γ production by stimulated human T cells (62). PGE₂ also induces apoptosis of thymocytes and inhibits T cell function, such as the production of IL-2 (63). In vivo, PGE₂ was shown to suppress the production of Th1 cytokines such as IL-12 (64). A recent study demonstrated that treatment with NS-398, the specific COX-2 inhibitor, promoted a Th1 immune response in BALB/c mice (65). In the same model, RSV infection increased Th2-type cytokines and increased CD4⁺ T cells in BAL fluid from challenged animals immunized with formalin-inactivated RSV vaccine (1). Taken together, these data suggest that excessive COX-2-mediated production of PGE₂ during RSV infection could potentially harm the host by repressing a desirable Th1 response while promoting an undesirable Th2 response.

Because both PGs and leukotrienes (LT) are clinically important proinflammatory molecules in the lung, and they both are products of arachidonic acid, inhibiting one pathway (in this case, PGs) would be anticipated to result in changes in the other pathway (LT) in the host. RSV infection induces the expression of 5-lipoxygenase, a key enzyme in the production of LTs from arachidonic acid (66). Determination of changes induced by the expression of COX-2 and 5-lipoxygenase during RSV infection will be important for understanding the dynamic interplay between PG and LT pathways and for the optimization of therapies that target these pathways.

Vaccine programs spanning 4 decades have yet to result in a safe and effective RSV vaccine. A major breakthrough in RSV...
prophylaxis was the development and licensure of both polyclonal and monoclonal Ig products for use in high risk infants (RespiGam (RSV Ig) and Synagis (palivizumab); MedImmune). Although highly effective in a preventive setting, reducing the hospitalization of high risk infants by up to 80% (22), Ig has not achieved success as a therapeutic agent. Despite the fact that Ig-treated patients showed a reduction in virus titers (67, 68), there was no clinically beneficial outcome from the treatment (67–70). Similar observations were made in the cotton rat, in which treatment of infected animals with palivizumab exclusively eliminated RSV from the host, but had no significant effect on pulmonary histopathology (41). Successful treatment of RSV bronchiolitis and pneumonia, however, was possible when a combination of antiviral (palivizumab) and anti-inflammatory (glucocorticoids) drugs was used in cotton rats (41). Thus, the histopathology induced by RSV infection is now largely attributed to the inflammatory response to infection. Glucocorticoids, however, are nonspecific immunosuppressors whose use is generally discouraged during infectious diseases, particularly in children. Study of the roles of the multiple inflammatory components regulated during RSV infection will potentially open the door to new and possibly more specific therapeutic strategies. One of these components, COX-2, has not been potentially open the door to new and possibly more specific therapeutic strategies. One of these components, COX-2, has not been.

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Disclosures

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