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ARTICLES

Meconium Aspiration Stimulates Cyclooxygenase-2 and Nitric Oxide Synthase-2 Expression in Rat Lungs

JAAKKO KYTÖLÄ, PEKKA KÄÄPÄ, AND PEKKA UOTILA

Research Centre of Applied and Preventive Cardiovascular Medicine [J.K., P.K.], Departments of Pediatrics [J.K., P.K.], Physiology and Clinical Physiology [P.U.], University of Turku, FIN-20520 Turku, Finland

ABSTRACT

To study the impact of meconium aspiration on the biosynthesis of prostaglandins and nitric oxide, we investigated the effects of intratracheal meconium instillation on the expression of cyclooxygenase-1 (COX-1) and -2 (COX-2) and endothelial (NOS-3) and inducible (NOS-2) nitric oxide synthase in rat lungs. Anesthetized, tracheotomized, and ventilated rats received 3 mL/kg human meconium suspension intratracheally ($n = 19$), and 14 control rats received an equal volume of saline. Ten rats were pretreated with indomethacin, and 13 rats were pretreated with dexamethasone. The lungs were ventilated with 70% oxygen for 3 h after the insult, and the level of COX-1, COX-2, NOS-2, and NOS-3 mRNA in lung tissue was analyzed by Northern blot hybridization. Furthermore, the expression and localization of the enzyme proteins was analyzed by immunohistochemistry. COX-1 and NOS-3 were clearly expressed in the lungs of control rats, whereas the level of COX-2 and NOS-2 expression was minimal. Meconium administration did not affect the expression of COX-1, but COX-2 expression was up-regulated in the respiratory epithelium and alveolar macrophages. Meconium also induced up-regulation of NOS-2 in the pulmonary epithelium, vascular endothelium, and macrophages. Indomethacin pretreatment did not affect the enzyme expressions, whereas dexamethasone administration significantly inhibited the meconium-induced COX-2 and NOS-2 up-regulation. Our data thus indicate that intrapulmonary meconium up-regulates lung COX-2 and NOS-2 gene expression, suggesting an important role for prostaglandins and nitric oxide in the meconium aspiration-induced pulmonary inflammation and hemodynamic changes. **(*Pediatr Res* 53: 731–736, 2003)**

Abbreviations

COX, cyclooxygenase
NO, nitric oxide
NOS, nitric oxide synthase

Aspiration of meconium frequently causes a severe neonatal disorder, characterized by initial airway obstruction with subsequent alveolocapillary injury and ventilation/perfusion mismatching in the lungs (1). In a few hours, the initial insult is followed by an inflammatory reaction in the lungs with neutrophil accumulation and concomitant increase in the pulmonary vascular resistance (1–4). The pathogenetic mechanisms of these alterations are still unclear, although the stimulation in the production of bioactive mediators in alveolar macrophages has been proposed (4–6).

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Correspondence: Jaakko Kytölä, M.D., Research Centre of Applied and Preventive Cardiovascular Medicine, University of Turku, Kiinamyllynkatu 10, FIN-20520 Turku, Finland; e-mail: jaakko.kytola@utu.fi
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Prostaglandins have been implicated as important modulators of various pathologic inflammatory and cardiovascular processes. The rate-limiting step in prostaglandin biosynthesis is the conversion of arachidonic acid to endoperoxides by cyclooxygenase enzymes, known to exist in two isoforms (7). The constitutive cyclooxygenase-1 (COX-1) is ubiquitously expressed and enrolled in physiologic processes, whereas the low basal activity of cyclooxygenase-2 (COX-2) can be stimulated by endogenous and exogenous mediators (7, 8). Accordingly, COX-2 is activated in endotoxin and cytokine-stimulated alveolar macrophages and pulmonary epithelial cells *in vitro* and this will result in the generation of large amounts of prostaglandin E_2 (8–10). The activity of pulmonary prostaglandin synthesis after aspiration of meconium is still poorly known, although our recent data indicate stimulated pulmonary expression of COX-2 upon exposure to meconium (11).

Nitric oxide (NO) is formed from L-arginine by nitric oxide synthase (NOS), which is expressed in several isoforms in a wide variety of cells. Similar to COX enzymes, the constitutive, endothelial NOS (NOS-3) is present under normal physiologic conditions, whereas the inducible NOS (NOS-2) is expressed in an inflammatory setting in the presence of endotoxin and cytokines (12). Although potentially protective, NO production by NOS-2 may also play a role in the induction of acute lung injury (13, 14). Furthermore, increased endothelial NO production may play an important role in pulmonary vascular adaptation at birth, and its inhibition may result in elevated pulmonary vascular resistance (15). In meconium aspiration, often complicated by pulmonary hypertension, the pulmonary synthesis of NO is, however, still poorly known.

The aim of our study was to investigate the effects of intratracheal instillation of meconium on the expression of COX and NOS genes in the lung tissue. To avoid the influences of postnatal adaptation on pulmonary gene expressions, we performed our studies in mature lungs. Furthermore, because COX and NOS pathways are shown to have interactions and prostaglandin production modulates induced NOS (16), we also assessed the effects of a COX inhibitor, indomethacin, and an inhibitor of both pathways, dexamethasone, on COX and NOS expression in meconium-instilled lungs.

METHODS

Animal preparation. Male Sprague Dawley rats ($n = 33$) that weighed 250–280 g were anesthetized with pentobarbital (60 mg/kg i.p., Mebunat, Orion-Farmos, Turku, Finland). The trachea was intubated through an incision in the neck. The lungs were ventilated with a pressure-controlled ventilator (Bourns BP 200 infant pressure ventilator, Riverside, CA; ventilatory rate 30 cycles/min, peak inspiratory pressure 15 cmH_2O, positive end-expiratory pressure 1–2 cmH_2O). The experiments were approved by the Committee of Animal Care in Research of the University of Turku.

Study protocol. Human meconium was collected from the first stools of several healthy term infants. It was frozen, pooled, lyophilized, and irradiated, and before the experiments it was diluted with sterile saline solution to a 65 mg/mL suspension. After tracheostomy, 3 mL/kg meconium suspension was instilled intratracheally into the lungs of 19 rats, and 14 control rats received an equal volume of saline. Five meconium and five saline rats received indomethacin (10 mg/kg i.p.) 15 min before the insult, and seven meconium and four saline rats were similarly pretreated with dexamethasone (1 mg/kg i.p.). After the instillation of meconium or saline, all lungs were ventilated with 70% oxygen for 3 h, and thoracotomy was then performed and a blood sample was taken from the left ventricle. Addition of 70% oxygen was chosen to avoid meconium aspiration–induced local hypoxia, which could have an effect on COX or NOS gene expressions. The pulmonary artery was cannulated and the left atrium was incised, and 20 mL of saline was infused into the pulmonary circulation to remove blood. The lungs were finally removed, and one lung of each animal was immediately homogenized in 5 volumes of denaturing solution containing guanidinium thiocyanate and kept at −70°C until analyzed. The other lung was fixed in buffered formalin.

Northern blot analysis. The total RNA of the lungs was extracted, size-fractioned, and transferred onto nylon membranes (Hybond-N, Amersham, Buckinghamshire, UK). The mRNAs of COX-1 and COX-2 were analyzed by specific cRNA probes as described earlier (11, 17). The mRNAs of NOS-2 and NOS-3 were analyzed using cDNA probes, which were generated by Prime-a-Gene Labeling System (Promega, Madison, WI, U.S.A.) using random primers and a 32P-dCTP (Amersham). The cDNA templates for mouse NOS-2 and human NOS-3 cDNA probes were from Cayman Chemical (Ann Arbor, MI, U.S.A.). These cDNA probes are suitable for the analysis of the corresponding rat mRNAs. The mRNA level of glyceraldehyde phosphate dehydrogenase was analyzed with a rat cDNA probe and was used as a reference (18), indicating equal loading of mRNA.

Immunohistochemistry. Formalin-fixed and paraffin-embedded rat lung specimens were cut into 5-μm sections, which were deparaffinized and blocked for endogenous peroxidase activity by 1% hydrogen peroxide in methanol for 15 min. Rehydrated sections were microwaved in 0.01 M sodium-citrate buffer (pH 6.0) for antigen retrieval. The avidin-biotin immunolabeling was carried out using Histostain-Plus Kit (Zymed, San Francisco, CA, U.S.A.) and monoclonal primary antibodies against ovine COX-1 and human COX-2 (Cayman Chemical) and against mouse NOS-2 and human NOS-3 (Transduction Laboratories, Lexington, KY, U.S.A.). These antibodies are suitable for the detection of corresponding compounds also in rat tissues. The tissue sections were treated with the blocking reagent for 20 min and incubated with the primary antibodies (dilution 1:200) overnight at 4°C. After subsequent washes with PBS (pH 7.5), the sections were treated with biotinylated secondary antibody containing 2% of normal rat serum for 20 min, washed, and treated with streptavidin-enzyme conjugate. The specimens were stained with diaminobenzidine and counterstained with Mayer's hematoxylin. Finally, the slides were dehydrated and mounted. Negative controls included slides from each lung specimens, which were treated with 1% normal goat serum in PBS instead of primary antibodies. No staining was detected in any negative controls without primary antibodies.

Data analysis. Data are presented as mean values ± SEM. Groups were compared using one-way ANOVA. When the overall ANOVA was significant, post hoc comparison between the groups was made using Student-Newman-Keuls test. Differences were considered significant at $p < 0.05$.

RESULTS

Meconium administration with or without pretreatments resulted in respiratory acidosis, but low arterial oxygen tension was observed only in two rats with meconium and indomethacin administration (Table 1).

COX-1 mRNA was clearly expressed in the control lungs, whereas the rate of COX-2 mRNA expression was negligible (Fig. 1). Meconium administration up-regulated COX-2 mRNA expression in the lungs, whereas the level of COX-1

Table 1. *Arterial pH, blood gases, and base excess in rats 3 h after intratracheal instillation of human meconium or saline*

	Saline ($n = 5$)	Saline + Indo ($n = 5$)	Saline + Dexa ($n = 4$)	Meconium ($n = 5$)	Meconium + Indo ($n = 5$)	Meconium + Dexa ($n = 9$)
pH	7.44 (0.04)	7.35 (0.05)	7.37 (0.04)	7.20 (0.04)*	7.21 (0.04)*	7.25 (0.04)*
Po_2	30.7 (3.9)	26.3 (6.3)	39.1 (4.7)	15.1 (2.1)	11.4 (3.5)	17.5 (5.9)
Pco_2	5.3 (0.6)	6.9 (0.8)	6.2 (1.1)	11.3 (1.2)*	10.4 (1.3)*	8.0 (0.5)
BE	2.0 (0.6)	1.3 (0.6)	0.7 (1.2)	−0.1 (0.7)	0.9 (0.6)	−2.7 (2.5)
So_2 %	99.6 (0.2)	97.6 (1.7)	99.7 (0.1)	95.4 (1.9)	70.5 (17.1)	81.5 (8.5)

Data shown as mean (SEM). Indo, indomethacin 10 mg/kg i.p. pretreatment; Dexa, dexamethasone 1 mg/kg i.p. pretreatment. * $p < 0.05$ vs saline.

mRNA was not changed (Fig. 1). Indomethacin pretreatment did not affect the expression of COX-1 mRNA or the up-regulation of COX-2 mRNA (Fig. 1). Dexamethasone had no effect on the level of COX-1 mRNA but inhibited the up-regulation of COX-2 mRNA stimulated by meconium (Fig. 2).

Immunohistochemistry showed that COX-1 protein was found in the respiratory epithelium, vascular endothelium, and alveolar macrophages in both control and meconium-treated lungs (Fig. 3*A*). COX-2 immunostaining was seen in control lungs only in some macrophages in the respiratory tract and alveolar space (Fig. 3*B*). After meconium instillation, increased immunostaining for COX-2 protein was detected mainly in the alveolar macrophages (Fig. 3*E*).

NOS-3 mRNA was clearly expressed in the control lungs, whereas the expression of NOS-2 mRNA was minimal (Fig. 1). NOS-3 protein was detected in the respiratory epithelium and vascular endothelium, as well as in alveolar macrophages of the control lungs (Fig. 3*F*). Meconium instillation did not change the level of NOS-3 mRNA but increased the expression of NOS-2 mRNA in the lungs (Figs. 1 and 2). Indomethacin pretreatment had no significant effect on the up-regulation of NOS-2 mRNA, whereas dexamethasone prevented the up-regulation of NOS-2 by meconium (Figs. 1 and 2). Indomethacin or dexamethasone itself had no effect on the level of either NOS-2 or NOS-3 mRNA (Figs. 1 and 2).

Only weak immunostaining for NOS-2 was detected in some bronchial and alveolar macrophages and capillary endothelium of control lungs (Fig. 3*G*). After meconium instillation, clear staining for NOS-2 protein was seen in the respiratory epithelium, vascular endothelium, and alveolar macrophages (Fig. 3*H*).

DISCUSSION

Several lines of clinical and experimental evidence suggest that inflammation with intrapulmonary accumulation of neutrophils is intimately involved in the pathogenesis of acute hypertensive lung injury in neonatal meconium aspiration (1–4). Although the mechanisms that initiate the intense pulmonary inflammation in a few hours after exposure to meconium are still poorly identified, intrapulmonary meconium may activate lung inflammatory cells to produce cytokines and thereby stimulate the arachidonic acid release and generation of proinflammatory and vasoactive eicosanoids in the lungs (4–6). We bring here evidence showing that intratracheal instillation of human meconium induces COX-2 and NOS-2 gene expressions in rat lungs without any effects on COX-1 or NOS-3. This effect seems to be mainly due to stimulation of alveolar macrophages, the predominant inflammatory cells in the alveoli, but the induction also occurs in epithelial cells of the respiratory tract and endothelial cells of blood vessels. Although the experimental lung injury in the present work was induced in fully developed lungs and the results therefore cannot be applied directly to the neonatal situation, the COX and NOS pathways seem to play a critical role in the normal but also in the deviating adaptation of the newborn lungs (15). However, our experimental approach enables us to study the gene expressions in lungs without the influence of the perinatal developmental regulation.

COX-dependent pathways seem to be involved in a variety of physiologic and pathophysiologic processes of the lungs. COX-1 is participated in production of prostaglandins under physiologic conditions, whereas COX-2 is associated with cell activation and inflammation by several stimuli (7). Studies in rats (19) and humans (9) indicate that lipopolysaccharide-stimulated alveolar macrophages can release inflammatory and vasoactive prostaglandins. Accordingly, in lungs with meconium contamination, increased prostaglandin E_2 and thromboxane A_2 formation has been found (20, 21). Corroborating our present results, this stimulated prostaglandin production seems to be due to up-regulation of COX-2 in macrophages, the predominant inflammatory cells in the alveoli (9). In agreement, our previous data indicate that COX-2 expression is induced in human blood monocytes, precursors of the alveolar macrophages, upon exposure to meconium (22). Because COX-2 is recognized as an immediate-early gene, induction of COX mRNA is followed by *de novo* synthesis of the enzyme polypeptide and prostaglandins (23). This time-dependent increase in lung prostaglandin production may then contribute to the pulmonary inflammatory and vascular hypertensive reactions, which are important components of meconium aspiration syndrome in neonates (3, 4).

The mechanisms that activate COX-2 transcription in the lungs still remain poorly known, although up-regulation of this enzyme is responsive to a variety of inflammatory mediators (7). Because acute hypoxia may also increase COX-2 mRNA expression in rat lungs (24), we cannot totally exclude some influence of local hypoxia in meconium-contaminated lungs. This hypoxic effect seems unlikely, however, because all of the studied rats in the present investigation were well oxygenated. However, because endotoxin- and cytokine-stimulated COX activity is the target for nonsteroidal and steroidal anti-inflammatory drugs (19, 25, 26), we also studied their effects in meconium-contaminated lungs. Not surprising, indomethacin, a more potent inhibitor of COX-1 than COX-2 activity in

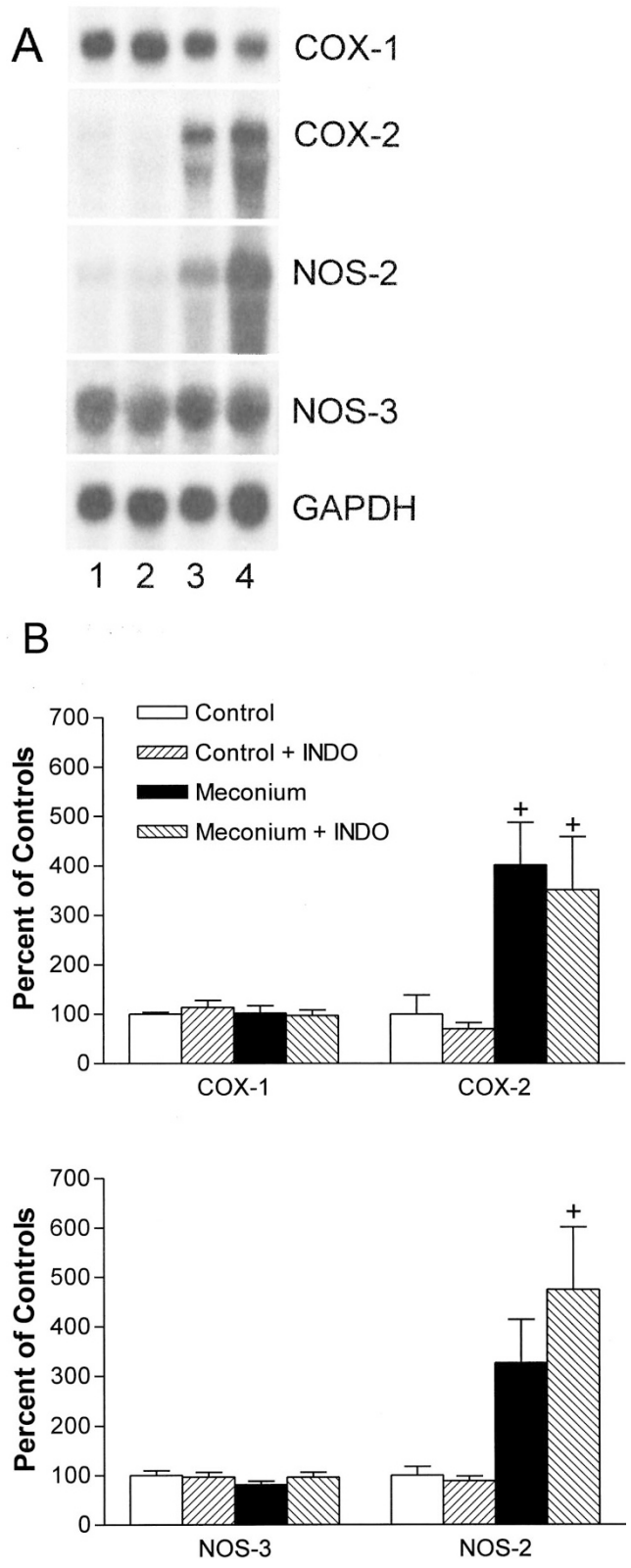

Figure 1. *A*, Northern blot analysis of COX-1, COX-2, NOS-2, NOS-3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs in saline-instilled control lungs (*lane 1*), saline-instilled lungs of rats pretreated with indomethacin (*lane 2*), meconium-instilled lungs (*lane 3*), and meconium-instilled lungs of indomethacin-pretreated rats (*lane 4*). *B*, The effects of meconium instillation and indomethacin pretreatment on the expression of COX-1, COX-2, NOS-3, and NOS-2 mRNA in rat lungs ($n = 5$ in each group). The mean level of mRNA in saline-instilled control lungs is expressed as 100% (±SEM). $+p < 0.05$, when compared with the controls.

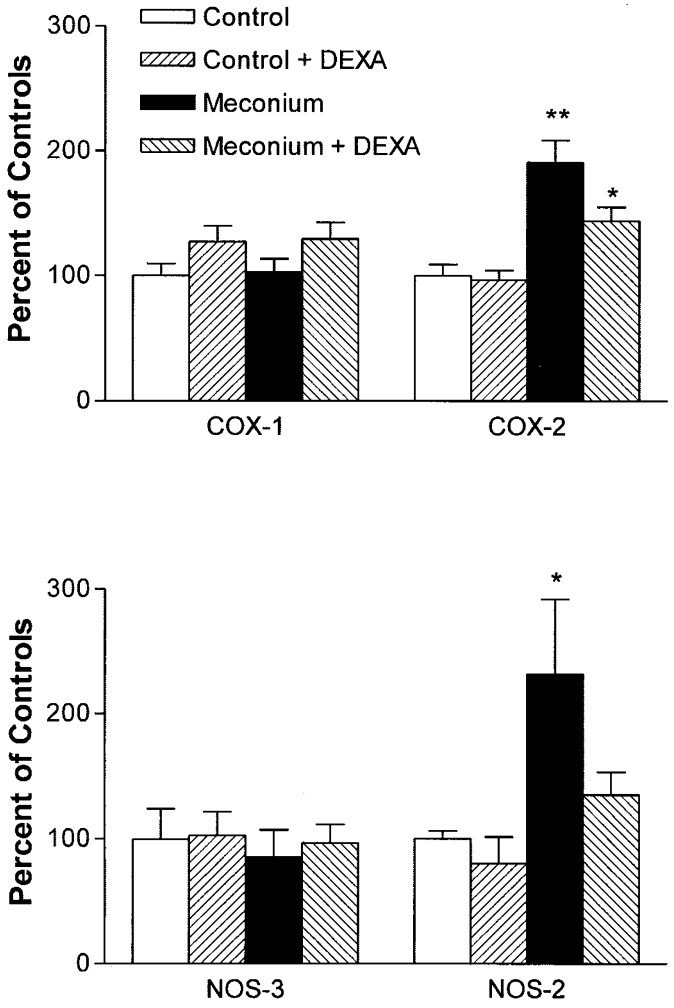

Figure 2. The effects of meconium instillation and dexamethasone pretreatment on the expression of COX-1, COX-2, NOS-3, and NOS-2 mRNA in rat lungs. The mean level of mRNA in saline-instilled control lungs is expressed as 100% (±SEM). $n = 5$ (control), $n = 4$ (dexamethasone-pretreated control group), $n = 5$ (meconium), $n = 9$ (dexamethasone-pretreated meconium group); $*p < 0.05$ *vs* all other groups; $**p < 0.01$ *vs* controls.

macrophages (25), did not prevent the meconium-induced COX-2 up-regulation in the lung tissue. Although we did not measure the end products of this enzymatic pathway, acetylsalicylic acid, a very potent COX-1 inhibitor, has been shown to prevent the postinsult rise in pulmonary prostaglandin synthesis after meconium aspiration (19, 21). However, previous dexamethasone administration, shown selectively to inhibit COX-2 transcription and activity in stimulated airway macrophages and epithelial cells (19, 26), significantly inhibited the up-regulation of this enzyme also in meconium-contaminated whole lungs of rats. Thus, although dexamethasone-dependent inhibition of COX-2 protein and prostaglandin synthesis involves significant posttranscriptional mechanisms (25), it is obvious that meconium-induced induction of COX, similar to endotoxin and cytokine stimulation (19, 23), occurs at the transcriptional level and supposedly thereby leads to modulation of the pulmonary inflammatory and vasoactive response.

Observations of the role of endogenous NO in acute lung injury are thus far contradictory. There is, however, evidence

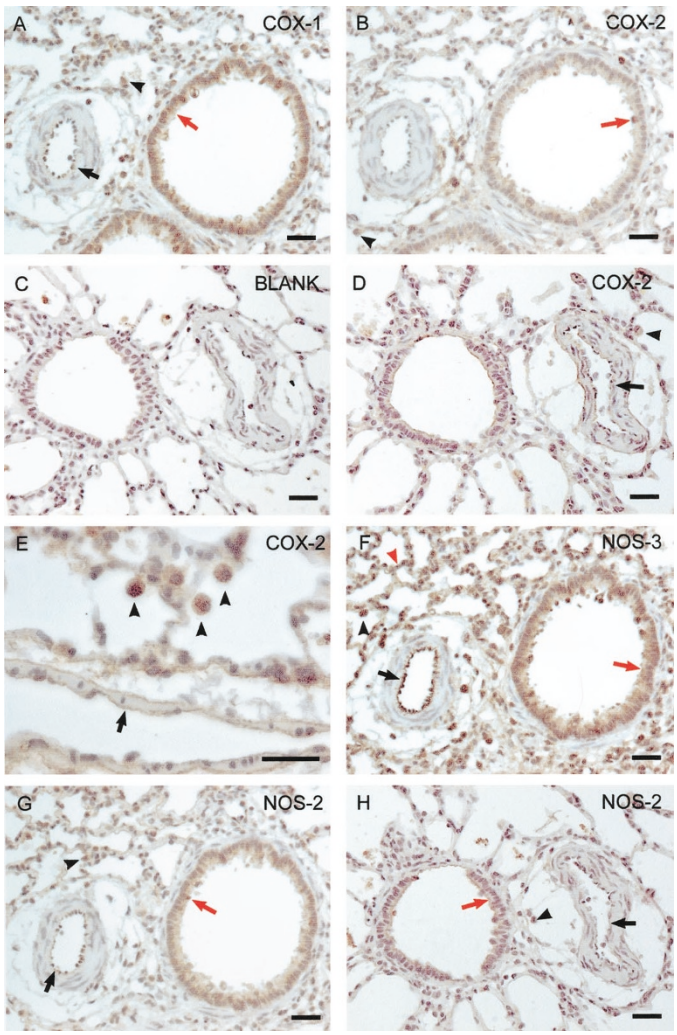

Figure 3. Immunoperoxidase staining of COX-1, COX-2, NOS-2, and NOS-3 protein in rat lungs 3 h after intratracheal instillation of saline (*A*, *B*, *F*, and *G*) or meconium (*C*, *D*, *E*, and *H*). *A*, Immunostaining for COX-1 is detected as brown color in the respiratory epithelial cells (*red arrow*), alveolar macrophages (*black arrowhead*), and vascular endothelial cells (*black arrow*) of a control rat. *B*, COX-2 immunostaining is detected only in some macrophages in the respiratory tract (*red arrow*) and the alveolar space (*arrowhead*) of control lungs. *C*, Negative control immunostaining of a meconium lung specimen (treated with normal goat serum instead of primary antibodies). *D* and *E*, After meconium aspiration, abundant staining for COX-2 is seen in alveolar macrophages (*arrowheads*) but not in the vascular endothelium (*arrow*). *F*, NOS-3 is amply stained in the bronchial epithelium (*red arrow*) and the capillary endothelium (*black arrow*) and moderately stained in the alveolar epithelium (*red arrowhead*) and the alveolar macrophages (*black arrowhead*) of a control lung. *G*, NOS-2 is weakly stained in the bronchial macrophages (*red arrow*), alveolar macrophages (*arrowhead*), and capillary endothelium (*black arrow*) of a control lung. *H*, After meconium aspiration, NOS-2 is clearly detected in the bronchial epithelial cells (*red arrow*) and in several alveolar macrophages (*black arrowheads*) and to some extent in the vascular endothelium (*black arrow*) but usually not in the alveolar epithelium. Scale bar = 30 μm.

showing that NOS-2 is expressed in a variety of pulmonary cells in response to proinflammatory cytokines and endotoxin and through increased endogenous NO production may lead to peroxynitrite formation and thereby aggravate the ensuing lung injury (13, 27, 28). Consistent with these findings, meconium may through stimulation of macrophages induce cytokine release and up-regulation of the pulmonary NOS-2 gene (5, 6). Further corroborating our data with meconium-contaminated lungs, dexamethasone but not indomethacin administration down-regulates NOS-2 expression in cytokine- and endotoxin-stimulated pulmonary tissues (27, 29, 30). It is thus conceivable that the ability of dexamethasone to inhibit NOS-2 expression directly may be one of the mechanisms by which glucocorticoids may oppose the injurious effects of aspirated meconium (31).

Interestingly, there are several lines of evidence suggesting mutual interference of the COX and NOS pathways (16, 29, 30). NO may have a regulatory role in the control of pulmonary COX-2 gene expression (16). Consequently, whereas endotoxin and cytokines promote NOS-2 and COX-2 expression in macrophages, inhibition of endogenous NO formation may decrease COX-2 mRNA (32, 33). Our present data suggest that, similar to endotoxin-induced stimulation, intratracheal meconium up-regulates both COX-2 and NOS-2 genes in lung tissue, and administration of dexamethasone significantly inhibits both pulmonary COX-2 and NOS-2 expressions (33). The data thus suppose that, in meconium-contaminated lungs, stimulated prostaglandin and NO production may play mutual modulatory roles in the inflammatory and hypertensive response.

CONCLUSION

In conclusion, intrapulmonary human meconium induces up-regulation of lung COX-2 and NOS-2 gene expression, suggesting an important role for prostaglandins and NO in the modulation of the pulmonary inflammatory and hypertensive injury induced by meconium aspiration. Inhibition of lung COX-2 and NOS-2 induction in response to meconium aspiration may be one of the mechanisms underlying the anti-inflammatory action of dexamethasone.

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