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Meconium Induces Expression of Inducible NO Synthase and Activation of NF-κB in Rat Alveolar Macrophages

YING-HUA LI, ZHONG-QUN YAN, ANNELIE BRAUNER, AND KJELL TULLUS

Neonatal Unit [Y.-H.L.] and Pediatric Nephrology [K.T.], Astrid Lindgren Children's Hospital, Karolinska Institute, Stockholm, Sweden; and Center for Molecular Medicine [Z.-Q.Y.] and Department of Clinical Microbiology [Y.-H.L., A.B.], Karolinska Hospital, Stockholm, Sweden

ABSTRACT

Meconium aspiration causes intensive inflammatory reactions in the lungs, and may lead to neonatal respiratory disorder. Infiltrated inflammatory cells, particularly macrophages, play an important role in such an inflammation. A rat alveolar macrophage cell line (ATCC8383) was exposed to meconium alone or in combination with dexamethasone, budesonide, or interferon-γ. Nitric oxide (NO) accumulation in the supernatant of the cell culture was detected by Griess reaction, and mRNA of inducible NO synthase (iNOS) expression was detected by reverse transcriptase-PCR. Nuclear factor-kappa B was analyzed by electrophoretic mobility shift assay, and iNOS location and nuclear factor-kappa B transactivation were determined by immunostaining. Our results showed that meconium was capable of inducing production of NO and expression of iNOS in alveolar macrophages in a dose- (1–25 mg/mL, $p < 0.05$) and time- (4–48 h, $p < 0.05$) dependent manner. This capability of meconium could be further enhanced in the presence of interferon-γ (100 IU/mL, $p < 0.05$). Budesonide (10^{-4}–10^{-10} M) or dexamethasone (10^{-4}–10^{-6} M) effectively inhibited the meconium-induced NO production ($p < 0.05$). Using the protein synthesis inhibitor cycloheximide, we demonstrated that meconium directly induced iNOS in macrophages. Furthermore, meconium also triggered nuclear factor-kappa B activation, a mechanism possibly responsible for the iNOS expression. Our findings suggest that meconium is a potent inflammatory stimulus, resulting in iNOS expression, leading to overproduction of NO from the macrophages, which may be of pathogenic importance in meconium aspiration syndrome. *In vitro* steroids down-regulated the iNOS expression, thus suggesting a potential to down-regulate NO-mediated inflammation in neonates with meconium aspiration syndrome. **(*Pediatr Res* 49: 820–825, 2001)**

Abbreviations:
LPS, lipopolysaccharide
RT-PCR, reverse transcriptase-PCR
NO, nitric oxide
iNOS, inducible nitric oxide synthase
IFN-γ, interferon-γ
NF-κB, nuclear factor-kappa B
CHX, cycloheximide
EMSA, electrophoretic mobility shift assay
MAS, meconium aspiration syndrome
NO_2^-, nitrite
G3PDH, glyceraldehyde-3-phosphate dehydrogenase

Meconium-stained amniotic fluid is frequently encountered during both term and postterm deliveries, and 1–3% of affected infants consequently develop MAS. This disorder is characterized by respiratory distress complicated with persistent pulmonary hypertension caused by airway obstruction and pneumonitis. MAS is a major cause of neonatal morbidity and mortality (1).

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Correspondence and reprint requests: Kjell Tullus, Astrid Lindgren Children's Hospital, Karolinska Institutet, SE-171 76 Stockholm, Sweden; e-mail: Kjell.Tullus@ks.se
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NO is an important molecule active in a number of biologic reactions (2, 3), especially with implications in inflammation (4). It is generated from L-arginine by three different NO synthases; of these, two are constitutive isoforms. The third is an inducible and Ca^{2+}-independent NO synthase (iNOS), normally produced only after transcriptional activation of its gene (5, 6). High levels of NO produced by iNOS can mediate lung injury (7). Laboratory studies have suggested that NO can potentiate the lung injury by promoting oxidative or nitrosative stress (8), inactivating surfactant, and stimulating inflammation (9).

The expression of iNOS is mediated by differential signaling transduction pathways. Among them, the NF-κB signaling pathway has been suggested as the determinant mechanism, for example, for cytokine production, regulation of adhesion mol-

ecules, and acute-phase protein synthesis (10). NF-κB may be induced by many endogenous and exogenous stimuli.

The purpose of this study was to investigate whether meconium could stimulate macrophages to produce NO, express iNOS, and activate NF-κB *in vitro* and further to evaluate the effect of dexamethasone and budesonide on NO production.

METHODS

Cell culture. A rat alveolar macrophage cell line (ATCC8383, Rockville, MD, U.S.A.) was maintained in Ham's F-12 medium (GibcoBRL, Gaithersburg, MD, U.S.A.) supplemented with 15% heat-inactivated fetal bovine serum Myclone (GibcoBRL).

Preparation of meconium. Meconium was collected from the first stools of three healthy newborn infants. It was pooled, resuspended in sterile PBS, filtered, and subsequently irradiated. No bacterial growth was found from meconium. Endotoxin levels in the meconium were <10 pg/mL as assessed by the *Limulus* amebocyte lysate endochrome test (Charles River Endosafe, Charleston, SC, U.S.A.). The sterile pooled suspension was then stored at −20°C for later use.

Antigen stimulation and effect of steroids on NO production. The rat alveolar macrophages were distributed into 24-microwell plates at a concentration of 1×10^6 cells/mL in serum- and phenol red–free medium. They were stimulated with either 1–25 mg/mL meconium, or 100 ng/mL LPS (O55:B5, Sigma Chemical Co., St. Louis, MO, U.S.A.) alone or in combination with 100 IU/mL IFN-γ (Genzyme, Cambridge, MA, U.S.A.) for 24 h at 37°C, in 5% CO_2. Meconium (5 mg/mL) was also incubated with the macrophages for different periods (4–48 h). For the effect of steroids on NO production, the rat alveolar macrophages were incubated with 5 mg/mL of meconium in combination with dexamethasone (10^{-4}–10^{-10} M) or budesonide (10^{-4}–10^{-10} M). To investigate whether meconium has a direct effect on NO production, the macrophages were incubated with 5 mg/mL of meconium in combination with the protein synthetase inhibitor CHX (1 μg/mL, Sigma Chemical Co.) for 24 h. Supernatants were collected after stimulation and stored at −70°C for analysis.

NO_2^- assay. The accumulation of NO_2^-, a stable end product of NO formation, in conditioned media was measured as an indicator of NO production. One hundred microliters of cell-free conditioned medium was incubated for 10 min with 100 μL of Griess reagent at room temperature, and the absorbance at a wavelength of 540 nm was automatically measured in a microplate reader. NO_2^- in the samples was calculated using a standard curve of sodium nitrite.

RT-PCR. Total RNA was extracted from cells with RNAzolB (Biotecx Laboratories; Houston, TX, U.S.A.) according to the manufacturer's instructions. First-strand cDNA synthesis of total RNA was performed using SuperScript RNase H^- Reverse Transcriptase (GibcoBRL) and random hexamer primers [$pd(N)_6$; Amersham Pharmacia Biotech, Uppsala, Sweden]. Specific oligonucleotide primers were synthesized for rat iNOS (Clontech, Palo Alto, CA, U.S.A.). The sequences of the 3'- and 5'-primers used are 5'-CCCTTCCGAAGTTTCTGGCAGCAG-3' and 5'-GGGCTCCTCCAAGGTGTTGCCC-3' (11). The rat G3PDH primer (Innovagen, Lund, Sweden) sequences are 5'-CTCAAGATTGTCAGCAATGC-3' and 5'-CAGGATGCCCTTTAGTGGGC-3' (12). The PCR using *Taq* polymerase (final concentration, 0.025 U/μL; GibcoBRL) was performed in a final volume of 25 μL containing 2 μL of cDNA for iNOS and G3PDH in a DNA Thermocycler 480 (Perkin Elmer, Norwalk, CT, U.S.A.) for 33 cycles for rat iNOS under the following conditions: 1 min denaturation at 94°C, 1 min annealing at 60°C, and 2 min extension at 72°C. PCR was conducted for rat G3PDH with 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. The PCR products were separated on a 1.5% agarose gel (GibcoBRL). The ethidium bromide–stained gel was photographed under UV light with the DC120 Digital Zoom Camera (Eastman Kodak, Rochester, NY, U.S.A.).

EMSA. Cells grown in serum-free medium were stimulated with meconium for 15, 30, and 60 min. Nuclear extracts were prepared as described (13), and nuclear protein concentrations were determined using the bicinchoninic acid kit (Pierce, Rockford, IL, U.S.A.). The nuclear extract (3 μg of protein) was preincubated for 10 min in the reaction buffer [10 mM HEPES, pH 7.9, 10% glycerol, 60 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 2 μg poly (dI-dC)], followed by incubation for 30 min at room temperature with 50,000 cpm of ^{32}P-labeled NF-κB probe (double-stranded oligonucleotides containing an NF-κB consensus binding site: 5'-AGTTGAGGGGACTTTCCCAGGC-3', Promega, Madison, WI, U.S.A.). After 30 min at room temperature, samples were separated on a 4% native polyacrylamide gel in low ionic strength buffer (22.3 mM Tris-borate, 0.5 mM EDTA, pH 8). Dried gels were autoradiographed with intensive screens at −80°C. In some cases, the incubation of nuclear extracts with ^{32}P-labeled NF-κB probe was performed in the presence of excess unlabeled NF-κB probe or the irrelevant oligonucleotide, AP-1 (Promega).

Transactivation of NF-κB and immunolocalization of iNOS. Cells (3000/well) were plated on glass coverslips and incubated with meconium, 5 mg/mL, 30 min for NF-κB and 24 h for iNOS. After treatment, the cells were fixed with cold methanol and acetone. Intracellular p65 and iNOS were visualized by indirect immunofluorescence using polyclonal rabbit anti-p65 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and polyclonal rabbit anti-macrophage iNOS antibodies (Affinity BioReagents, Golden, CO, U.S.A.), followed by FITC-labeled goat anti-rabbit IgG (Dako, Copenhagen, Denmark).

Data analysis. Data from pooled experiments were reported as the mean ± SEM NO_2^- concentrations (micromolar). Data were analyzed by Student's *t* test or one-way ANOVA. A $p < 0.05$ was considered to be significant.

RESULTS

NO production and iNOS expression. Meconium stimulated the production of NO from the alveolar macrophages in a dose- and time-dependent manner (Fig. 1). All tested concentrations >1 mg/mL meconium stimulated the production of NO compared with the control samples ($p < 0.05$) at 24 h. Five milligrams per milliliter meconium itself contains 5.06 ± 0.79

μM NO_2^-. The NO production was already stimulated after 4 h incubation with 5 mg/mL meconium ($p < 0.05$) and continuously increased during the studied 48 h ($p < 0.05$). The capability of meconium to stimulate NO production was further enhanced in the presence of IFN-γ (100 IU/mL, $p < 0.05$; Fig. 1*A*).

Because iNOS is regulated mainly at the transcriptional level, iNOS transcripts were examined by RT-PCR. Meconium stimulated macrophages to express higher levels of iNOS mRNA compared with untreated macrophages (Fig. 2). The iNOS protein was also shown in the meconium-stimulated cells with immunostaining techniques (Fig. 3*A*).

To detect whether meconium has a direct effect on iNOS expression, we used the protein synthetase inhibitor CHX to block the *de novo* synthesis of, for example, cytokines. As shown in Figure 2, the induction of macrophage iNOS expression by meconium was not dependent on cytokine production.

NF-κB expression. The effect of meconium on the NF-κB signal transduction pathway in the alveolar macrophages was determined by EMSA. No clear effect was seen after a 15-min treatment with meconium. After 30 and 60 min, the NF-κB binding complexes observed were substantially enhanced in the nuclear extracts of the macrophages (Fig. 4). The specificity of the NF-κB–DNA complex was ascertained by competition study. As shown in Figure 4, the indicated NF-κB–DNA complexes were removed by excessive cold NF-κB probe but were not affected by excessive AP-1 probe. The activated expression of NF-κB was also determined by immunostaining in terms of the translocation of p65 in cells treated by meconium for 30 min and in untreated cells. In the untreated macrophages, p65 was sequestered in the cytoplasm; whereas in the cells treated with meconium (5 mg/mL), translocation of p65 into the nuclei of the macrophages was seen (Fig. 3*B*).

Down-regulation of NO production by steroids. Budesonide (10^{-4}–10^{-10} M) and dexamethasone (10^{-4}–10^{-6} M) significantly inhibited NO production stimulated by meconium ($p < 0.05$; Fig. 5). Budesonide ($\geq 10^{-6}$ M) was more potent than dexamethasone at the same concentration ($p < 0.05$).

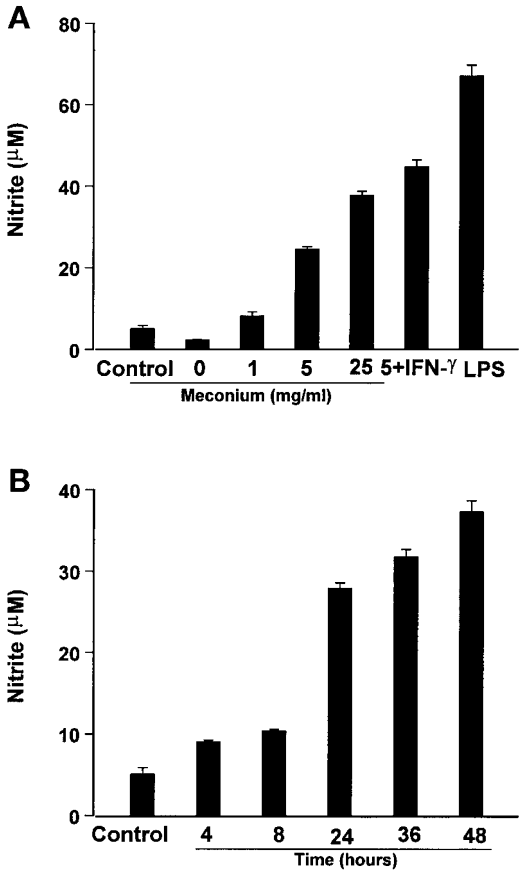

Figure 1. *A*, NO production after 24 h stimulation in the rat alveolar macrophage cell line with LPS (100 ng/mL), meconium (1–25 mg/mL), and meconium (5 mg/mL) in combination with IFN-γ (100 IU/mL). Control denotes the concentration of NO_2^- in 5 mg/mL meconium. NO activity was assessed by determining NO_2^- concentration in conditioned medium. *B*, NO production after stimulation with 5 mg/mL meconium at different times. NO production increases with time.

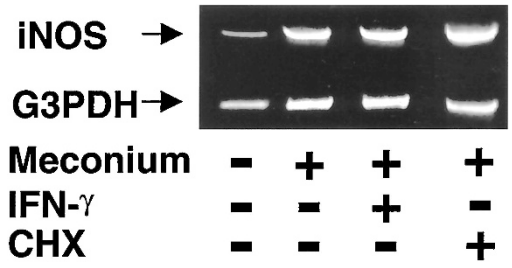

Figure 2. Expression of iNOS in the macrophages studied with RT-PCR after stimulation with 5 mg/mL meconium alone or together with IFN-γ (100 IU/mL) or CHX (1 μg/mL).

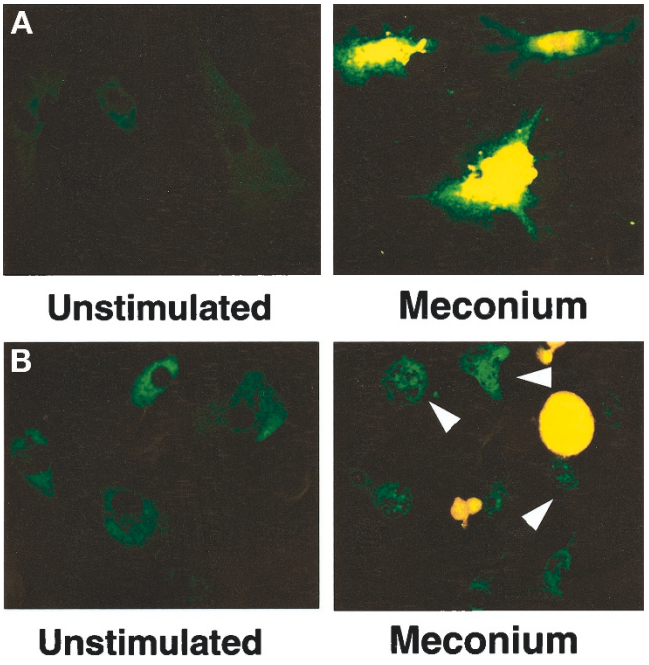

Figure 3. *A*, immunostaining of the rat alveolar macrophages for iNOS after stimulation with 5 mg/mL meconium compared with unstimulated cells. Original magnification ×500. *B*, immunostaining for NF-κB expression after 30-min incubation with 5 mg/mL meconium compared with unstimulated cells. The intracellular location of p65 was detected by indirect immunofluorescence with an anti-p65 antibody. *Arrowheads* indicate the transactivated NF-κB. Original magnification ×500.

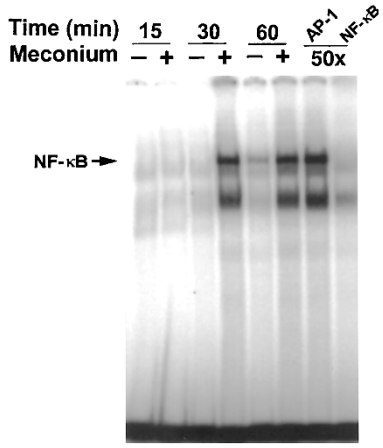

Figure 4. NF-κB binding complexes studied by EMSA. After treatment with meconium for 30 and 60 min, NF-κB binding complexes were substantially enhanced. No alteration was observed after 15 min. The indicated NF-κB–DNA complexes were removed by 50× excessive cold NF-κB probe but not by excessive AP-1 probe.

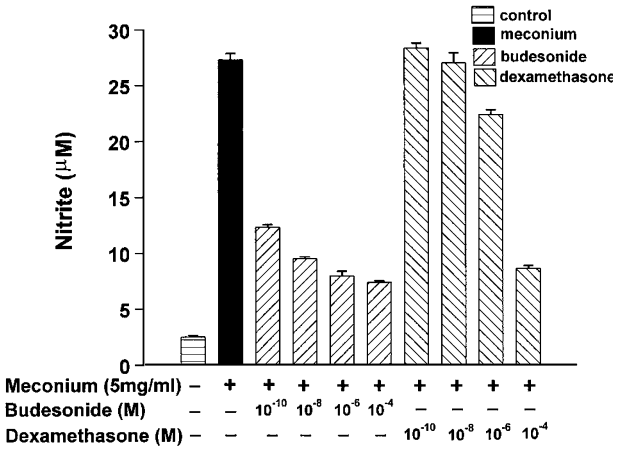

Figure 5. Down-regulation of 5 mg/mL meconium-stimulated NO production in the rat alveolar macrophage cell line by different doses of budesonide and dexamethasone.

DISCUSSION

We have, for the first time, shown that meconium could induce iNOS gene expression and lead to high output of NO from alveolar macrophages in a dose-dependent way. CHX did not affect the iNOS mRNA expression, indicating that meconium had a direct effect, not *via* cytokine production, on the NO production. Both budesonide and dexamethasone down-regulated the meconium-stimulated NO production. Likewise, NF-κB was also activated by meconium.

The mechanisms for the development of MAS are not completely understood. After meconium aspiration, an intense inflammatory response occurs, with polymorphonuclear lymphocytes found diffusely through the lungs. This inflammatory reaction, supposedly initiated by meconium-induced activation of pulmonary macrophages (14), is associated with increased pulmonary vascular permeability, leading to proteinaceous exudation into the alveolar spaces, and thereby inactivation of pulmonary surfactant and decreased lung compliance. *In vitro* studies have indicated that the neutrophils and plasma proteins accumulating in the alveoli as a result of the inflammatory response are potential inhibitors of surfactant (15), which may also explain why meconium can inactivate surfactant (16). It has previously been shown that meconium could stimulate IL-8 expression (17) and inhibit neutrophil oxidative burst and phagocytosis (18), and high levels of IL-6 in meconium-stained amniotic fluid have been detected (19). Meconium aspiration may also cause apoptosis in epithelial cells (20). All these inflammatory reactions, together with our present findings, may be part of the pathologic basis of MAS and lead to not only structural changes in the neonate, such as complete obstruction of the upper airways, atelectasis, pulmonary interstitial emphysema, or chemical pneumonitis, but also several physiologic changes, such as hypoxemia, hypercapnia, acidosis (21, 22), persistent pulmonary hypertension, and even death (23–25). There exist several mechanisms through which relatively high levels of NO produced by iNOS can mediate lung injury (7). Reaction of NO with superoxide anions produces peroxynitrite, which is a highly oxidative species that is capable of nitrating tyrosine residues of numerous proteins, leading to the formation of nitrotyrosine. High levels of nitrotyrosine formation have been shown to be involved in acute lung injury in humans and LPS-injected animals.

Clinical studies have suggested that NO is an important inflammatory mediator in critically ill patients (26–28), especially in neonatal fulminant early onset pneumonia (29). Likewise, monocytes and tissue macrophages isolated from patiens with rheumatoid arthritis, tuberculosis, and malaria display higher levels of iNOS and generate increased levels of NO *in vitro* (30). It is known that there are species differences of NO production. Weinberg (30) has reviewed the reports from 1989 to 1998 regarding NO production and iNOS expression in human mononuclear phagocytes in which there were some difficulties in detecting NO production, partly depending on the method used. Rodent mononuclear phagocytes have been used for many *in vitro* studies (31, 32), mainly because they are more sensitive.

NO has now acquired considerable notoriety as a representative of a new class of messenger molecules that are responsible for various functions in many different tissues. Although the physiologic production of NO plays a key role in the host-defense response against various intracellular pathogens, its overproduction may be responsible in part for the pathophysiology of infection (33). NO is produced by cells responsible for a number of different functions in the vascular endothelium (34), cells of the immune system (35), smooth muscle (36), and cardiac muscle (37). High-output NO may cause increased permeability of vascular endothelial cells, inhibit leukocyte adhesion, degrade carbohydrates, inhibit lipid peroxidation, and cleave DNA *via* nitrosation, nitration, and oxidation, and thus may lead to the pathophysiologic changes in MAS.

There are reports of clinically beneficial effects of NO inhalation with some limitations and side effects. Nitric oxide therapy seems to reduce the need for extracorporeal membrane oxygenation (38), but has no apparent effect on mortality in

critically ill infants with hypoxic respiratory failure (39). Treatment with high-frequency oscillatory ventilation plus inhaled NO is often more successful than treatment with high-frequency oscillatory ventilation or inhaled NO alone in severe persistent pulmonary hypertension of the newborn (40). Early continuous NO inhalation controls the rise in pulmonary artery pressure and improves the efficiency of arterial oxygenation, and further prevents the increase in epithelial apoptosis, but does not protect against early inflammatory damage caused by meconium aspiration (41). In the newborn pig model of MAS, short-term exposure to inhaled NO does not decrease pulmonary artery pressure or improve oxygenation (42). The dual effects of NO, the difference between exogenous and endogenous NO, the functional site of NO, and the local concentration of NO need to be further investigated.

NF-κB is known to be a ubiquitous rapid-response transcription factor expressed in a wide variety of cells and involved in the generation of a number of inflammatory gene products (10). A number of endogenous and exogenous stimuli can induce NF-κB activation. The NF-κB site in the iNOS promoter region is essential for LPS- or oxidative stress-induced NO production (5). The role of NF-κB in iNOS gene expression has been well elucidated. Stimulation of macrophages with LPS or other cytokines leads to activation of NF-κB and subsequently binding to the κB response element of the iNOS promoter. It has, however, been unclear whether meconium is able to activate NF-κB. Our data demonstrate that meconium is a potent activator of NF-κB, as evidenced by our finding that meconium could trigger a rapid and intense NF-κB activation in macrophages. This suggests that NF-κB activation may be a key mechanism responsible for the meconium-induced iNOS expression pathway. In addition, the potential role of NF-κB in inflammation and immune modulation in MAS is not limited to transcriptional activation of iNOS. In fact, NF-κB has been shown to exert a crucial role in the inducible expression of many inflammatory genes encoding transcriptional factors, adhesion molecules, cytokines, and growth factors (43). Therefore, meconium-induced NF-κB activation in macrophages may represent a key mechanism responsible for the inflammatory reaction associated with MAS.

Management of MAS patients is with mechanical ventilation and the administration of exogenous surfactant or extracorporeal membrane oxygenation (44). Despite significant advances, MAS still causes important morbidity and mortality. Budesonide (an inhaled steroid) and dexamethasone (a systemic steroid) have been used to inhibit the inflammatory response, and they can down-regulate the proinflammatory cytokine production *in vitro* (45, 46). The present study shows that they also inhibited the meconium-stimulated NO production and suggests that steroids could be used in the treatment of MAS.

In conclusion, our findings show that meconium is a potent inflammatory stimulus, resulting in iNOS expression, leading to overproduction of NO from the macrophages, which could result in MAS.

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