Meconium aspiration increases iNOS expression and nitrotyrosine formation in the rat lung

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Background: Despite it is recognized that high level of nitrotyrosine formation may play a role in acute lung injury (ALI), few studies have assessed protein nitration in ALI after meconium aspiration. This study was undertaken to observe the expression of inducible nitric oxide synthase (iNOS) and formation of nitrotyrosine in ALI after meconium aspiration and evaluate the contribution of iNOS and nitrotyrosine to tissue injury.

Methods: Sixteen healthy male Sprage-Dawley rats were divided randomly into control group and meconium group, which were administrated intratracheally 1 ml/kg saline or 20% human newborn meconium suspension respectively. The animals were sacrificed 24 hours after treatment. Bronchoalveolar lavage fluid (BALF) cell count, BALF protein, pulmonary myeloperoxidase (MPO) activity, malondialdehide (MDA), and nitrate/nitrite levels were measured. Western blot was used to determine the expression of pulmonary nitrotyrosine, a specific "footprint" of peroxynitrite and iNOS. Lung injury score was also evaluated.

Results: Compared with the control group, the rats in the meconium group showed an increase in cell count (mean±SD 4.04±1.01 vs 0.53±0.19×10^6/ml, P<0.01), BALF protein (mean±SD 2.5±0.74 vs 0.67±0.26 mg/L, P<0.01), pulmonary MPO activity (mean±SD 1.49±0.22 vs 0.62±0.16 U/g wet lung, P<0.01), MDA level (mean±SD 3.30±0.85 vs 1.40±0.35 nmol/mg protein, P<0.01), nitrate/nitrite level (mean±SD 12.77±5.00 vs 4.89±1.32 µmol/mg protein, P<0.01), and lung injury score (9.88±1.36 vs 2.25±1.04, P<0.01). Western blot examination demonstrated increased expression of nitrotyrosine and iNOS in the meconium group (mean±SD 0.46±0.19 vs 0.11±0.08 and 1.49±0.60 vs 0.13±0.11, respectively, P<0.01).

Conclusions: Meconium causes increased expression of pulmonary iNOS, leading to over production of NO and nitrotyrosine, which may be of pathogenic importance in ALI after meconium aspiration.

Key words: meconium; lung injury; nitrotyrosine; nitric oxide synthase

Introduction

Meconium aspiration syndrome (MAS) remains one of the principal causes of neonatal respiratory distress, frequently leading to respiratory failure, persistent pulmonary hypertension of the newborn (PPHN) or death.[1] Meconium-induced lung injury is associated with many pulmonary changes that contribute to respiratory failure, including airway obstruction, inflammatory cell infiltration with the release of bioactive mediators and surfactant dysfunction. It is firmly established that pulmonary interstitial inflammation and inactivation of surfactant are the underlying causes of acute respiratory distress syndrome (ARDS) and pulmonary hypertension.[2-4] Many animal and human studies focused on the efficiency of exogenous surfactant in treating meconium aspiration[5-7] and pulmonary inflammation with meconium,[8-10] but the mechanism of surfactant inactivity in meconium-induced respiratory failure is not clear.

Previous study in vitro has shown that meconium is able to induce mRNA expression of inducible nitric oxide synthase (iNOS) followed by a high level of NO production.[10] Reaction of NO with superoxide anions produces peroxynitrite (ONOO^-), which is a highly oxidative species capable of nitrating tyrosine residues of numerous proteins, leading to the formation of nitrotyrosine. Studies have suggested that an increased endogenous NO production can potentiate lung injury by promoting oxidative or nitrosative stress, stimulating
inflammation, and inactivating surfactant.\textsuperscript{[11-13]} The relationship of ONOO\textsuperscript{-} with acute lung injury (ALI) has been the focus of numerous animal or human studies. Despite several studies suggested that the high level of nitrotyrosine formation plays a role in ALI, few studies have assessed protein nitration in ALI after meconium aspiration.

Furthermore, pulmonary inflammation with meconium aspiration is peaked during 16-24 hours.\textsuperscript{[6]} Mechanical support with oxygen may also induce lung inflammation and injury. To better understand the pathogenesis of acute lung injury caused by meconium, we established a rat model of meconium-induced ALI with neither O\textsubscript{2} supplement nor mechanical support and observed the changes of pulmonary inflammatory response, formation of nitrotyrosine and expression of iNOS in 24 hours after meconium aspiration.

In this study, we hypothesized that meconium would increase pulmonary NO and nitrotyrosine by inducing the expression of pulmonary iNOS.

**Methods**

**Meconium preparation**

The first-passed meconium taken from urine-free diapers of normal term infants was placed in sterile jar and frozen for less than 7 days and then lyophilized, pooled, and diluted with sterile saline solution to a final concentration of 20\% suspension. This meconium slurry was stored in 2 ml aliquots and frozen at -80\(^\circ\)C until use.

**Animal management**

Sprague-Dawley rats were obtained from the Animal Center of the Zhejiang Medical Institute, Hangzhou, China. Healthy male rats of 130-170 g (30-45 days old) were utilized for the present experiments. Meconium was administered to the respiratory tract of rats as the described previously.\textsuperscript{[8]} Briefly, the rats were slightly anesthetized after intraperitoneal injection of 40 mg/kg pentobarbital. A small midline incision was made on the ventral aspect of the neck to expose the trachea, while an endotracheal tube was placed through the incision. The tube was then stabilized. Prior to meconium instillation, the rats were injected intraperitoneally 2 mg/kg vecuronium bromide. After 15 minutes, the rats were divided randomly into control group and meconium group (\(n=8\), each). Afterwards, 1 ml/kg sterile saline or 20\% meconium was instilled through the endotracheal tube, followed by a 3 ml bolus of air to disperse the meconium into the lung. Half the dose was given with the rat lying on one side and half with the rat lying on the other side. Then, skin and tracheal incisions were closed with 4-0-nylon suture and all the rats were allowed to breathe spontaneously in room air.

**Sample collection and storage**

Twenty-four hours after surgery, the rats were killed. The chest of the animals was opened by a midline incision, the lungs were isolated, and the lavage of the left lung was performed. Tissue samples from the right lung were obtained.

**Total cell count and protein concentration of bronchoalveolar lavage fluid (BALF)**

Through tracheostomy, the left lung was lavaged using three 1-ml aliquots of cold sterile saline, each of which was instilled and withdrawn 3 times, and then mixed. The recovered total BALF was always more than 90\%. A total cell count of mixed BALF was obtained within 4 hours. Protein concentration of BALF was determined by the Bradford protein assay.\textsuperscript{[14]}

**Pulmonary myeloperoxidase (MPO) activity**

As a measure of pulmonary neutrophil influx and activity, the MPO activity was assessed in duplicate on homogenized lung samples according to the manufacture's manual. One unit of the MPO activity was expressed as units per gram wet lung tissue.

**Pulmonary malondialdehide (MDA) assay**

Pulmonary MDA levels were assayed in duplicate on homogenized lung samples. The results were standardized for tissue protein concentration determined by the Bradford protein assay.\textsuperscript{[14]}

**Pulmonary nitrate/nitrite assay**

Pulmonary nitrate/nitrite levels were assayed in duplicate on homogenized lung samples according to the manufacture's manual. The results were standardized for tissue protein concentration determined by the Bradford protein assay.\textsuperscript{[14]}

**Western blot analysis of nitrotyrosine formation and iNOS protein expression**

Lung tissues were homogenized with 5 volumes of lysis buffer containing 1\% (vol/vol) NP-40, 0.5\% (wt/vol) sodium deoxycholate, and 0.1\% (wt/vol) sodium dodecyl sulfate (SDS) in phosphate-buffered saline (PBS), to which a protein inhibitor, phenylmethylsulfonyl fluoride
100 µg/ml, was added. The homogenate was left on
ice for 30 minutes before centrifugation at
10000 × g. Protein content was estimated, and aliquots of 50
µg of homogenate protein were mixed a ratio of 1:1
with 2×sample buffer containing 125 mmol Tris, 20%
w/vol) glycerol, and 4% (w/vol) SDS. Separated on
10% (w/vol) PAGE gel the mixture was transferred
to nitrocellulose-ECL membrane, and stained with
Coomassie blue to verify the equivalent transfer of
samples. The membrane was blocked by incubation
with 3% (v/vol) PBS or dry milk in PBS for 1 hour
at room temperature in shaking. Thereafter, the
membrane was incubated overnight at 4°C with either
mouse monoclonal anti-nitrotyrosine (Calbiochem-
Novabiochem Co., San Diego, USA) or rabbit
polyclonal anti-iNOS or anti-actin (Santa cluz, USA).
The primary antibodies were used at dilutions of 1:500,
1:1000 or 1:1000. The blots were washed four times (10
minutes each time) with Tris-buffer saline Tween-20
(TBST), comprising 50 mmol Tris-HCl (pH 7.6), 150
mmol NaCl and 0.1% Triton X-100, and incubated
subsequently for 2 hours at room temperature in Tris-
buffered saline with horseradish peroxidase-conjugated
anti-mouse or anti-rabbit IgG antibody (1:5000 dilution;
Pierce). Immunoreactive bands were visualized using
the enhanced chemiluminescence (ECL) system
(Pierce) and quantified by densitometric analysis, using
Scion image software. The results were calculated as a
relative ratio of specific band to the actin one.

**Histology**

For histological analysis of the lung, pulmonary tissues
taken from the right lobe were fixed in buffered
formalin. The tissues were dehydrated, embedded in
paraffin and selected. The sections in 5 µm thickness
were stained with hematoxylin and eosin for light
microscopic analysis. To determine the extent and
severity of lung tissue injury, the samples were
assessed by a pathologist blinded to the grouping of
rats. A score from 0 to 4 was assigned for each of
different characteristics: (i) the extension of
leukocyte infiltration: 0=0%, 1=0-25%, 2=25%-50%,
3=50%-75%, and 4=75%-100% of the section areas
occupied by leukocytes; (ii) the amount of intra-
alveolar leukocytes: 0=no, 1=occasional, 2=several
leukocytes in the alveoli; 3=alveoli almost full of
leukocytes, and 4=alveoli distended by leukocytes; and (iii) the amount of exudative debris,
including fibrin, hyaline membranes, edema fluid
and meconium: 0=alveoli open, 1=exudate scarcely
seen, 2=exudate clearly visible, 3=alveoli almost full
of exudate, and 4=alveoli distended by exudate. The
calculated total injury score was considered the sum of
these scores.

**Statistical analysis**
The data were expressed as mean ± standard deviation
(SD). Student’s t test was used to detect the difference
between the groups. The Mann-Whitney µ test was
used for the analysis of lung injury score. A P value
less than 0.05 was considered statistically significant.

**Results**

**Histological findings**
The control lungs were normal and essentially well
aerated (Fig. 1A). Meconium instillation induced a
marked inflammatory reaction. The alveoli contained
various amounts of leukocytes, fibrin and occasionally
epithelial cells of instilled meconium. The lungs from
the meconium group appeared to be edematous and
had patchy areas of hemorrhage (Fig. 1B). Lung injury
score increased more significantly in the meconium
than in the control group (mean±SD 9.88±1.36 vs
2.25±1.04, P<0.01, Fig. 1C).

**Pulmonary MPO activity and BALF cell count**
Pulmonary MPO activity, a marker of leukocyte
infiltration, was increased more significantly in the
meconium group than in the control group (mean
±SD 1.49±0.22 vs 0.62±0.16 U/g wet lung, P<0.01).
At the same time, the total cell count of BALF was
significantly increased in the meconium group (mean
±SD 4.04±1.04 vs 0.53±0.19×10^6 cells/ml, P<0.01)
(Table).

**BALF protein concentration**
Compared with the control group, the rats in the
meconium group were characterized by significantly
increased BALF protein concentration (mean±SD 2.54
±0.74 vs 0.67±0.26 mg/L, P<0.01) (Table).

**Pulmonary MDA and nitrate/nitrite level**
Compared with the control group, the rats in the
meconium group showed a marked increase in
pulmonary MDA level (mean±SD 3.30±0.85 vs 1.40±
0.35 nmol/mg protein, P<0.01) and nitrate/nitrite level
(mean±SD 12.77±5.00 vs 4.89 ±1.32 µmol/mg protein,
P<0.01) (Table).
Pulmonary nitrotyrosine and expression of iNOS

Western blot analysis of nitrotyrosine demonstrated a prominent band of nitrotyrosine and iNOS at approximately 60 kD and 115 kD, respectively. The intensity of both nitrotyrosine and iNOS bands was greater in the meconium group than in the control group, indicating that meconium-induced ALI increases the formation of pulmonary nitrotyrosine (0.46±0.19 vs 0.11±0.08, \(P<0.01\)) and the expression of pulmonary iNOS (mean±SD 1.49±0.60 vs 0.13±0.11, \(P<0.01\)) (Fig. 2).

Discussion

The results of the present study have shown that meconium aspiration is characterized by an increase in pulmonary MDA level (a marker of oxidative injury) and leakage alveolocapillary protein, and that meconium also induces pulmonary iNOS protein expression and overproduction of NO. For the first time, we found that nitrotyrosine, a marker of peroxynitrite production, is increased in patients with ALI due to meconium aspiration.

Meconium contains such noxious agents as cholesterol, free fatty acids and bilirubin, which may initiate inflammatory reaction in the lungs and interfere with surfactant function. Previous in vitro studies demonstrated that surfactant was inactivated by both water and chloroform-soluble fractions of meconium;\(^{16,17}\) but the pathogenic mechanisms of this alteration are not elucidated.

The mechanism of activated neutrophils in patients with ALI caused by meconium aspiration has been well investigated. Influx of neutrophils into the airspaces is a consistent feature of ALI after various direct and indirect insults. Besides neutrophils, pulmonary macrophages including intra-alveolar and pulmonary vascular macrophages were shown to be involved in the pathogenesis of ALI\(^{[4,18,19]}\). It was reported that meconium could induce iNOS gene expression and lead to a high output of NO from alveolar macrophages in a dose-dependent manner. Moreover, it has a direct effect, not via cytokine production, on the NO

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell count ((×10^6/ml))</th>
<th>Protein (mg/L)</th>
<th>MPO (U/g wet lung)</th>
<th>MDA (nmol/mg prot)</th>
<th>Nitrate/nitrite (µmol/mg prot)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meconium</td>
<td>4.04±1.01(^a)</td>
<td>2.54±0.74(^a)</td>
<td>1.49±0.22(^a)</td>
<td>3.30±0.85(^a)</td>
<td>12.77 ±5.00(^a)</td>
</tr>
<tr>
<td>Control</td>
<td>0.53±0.19</td>
<td>0.67±0.26</td>
<td>0.62±0.16</td>
<td>1.40±0.35</td>
<td>4.89 ±1.32</td>
</tr>
</tbody>
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\(^a\): vs the control group, \(P<0.01\).
of reactive nitrogen species (RNS)-mediated reactions. Therefore, it serves as footprints of RNS action. High levels of nitrotyrosine formation have been shown to be involved in ALI in humans and LPS-injected animals. It can diminish the function of a variety of crucial proteins, including pulmonary surfactant protein A (SP-A) and manganese superoxide dismutase.[22] The results of previous in vitro studies indicated that the nitration of human SP-A by ONOO\(^-\) inhibits its lipid aggregation and mannose binding activity. This may be one of the factors responsible for the increased susceptibility of patients with ARDS to nosocomial infections.[21,23]

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.[4,6] On the other hand, it is speculated that the nitration of surfactant protein may be another cause of surfactant inactivation from the results of this study.

In conclusion, instillation of human meconium into the animal lung induces up-regulation of lung iNOS protein expression, thus leading to the overproduction of NO and the formation of nitrotyrosine. Nitration of surfactant protein may be another cause of surfactant inactivation that is related to ARDS and pulmonary hypertension caused by meconium aspiration. The consequence of protein nitration in patients with ALI due to meconium aspiration remains to be determined.

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