Effect of TLR-4 and HO-1 on acute lung injury induced by hemorrhagic shock in mice

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Objective: To examine whether TLR-4 has an effect on hemorrhage induced changes in lung, and to investigate the change of heme oxygenase-1 (HO-1) on acute lung injury (ALI) induced by hemorrhagic shock in mice.

Methods: Forty-eight male mice, including C3H/HeN mice and C3H/HeJ mice, were randomly divided into sham group (n=12), hemorrhagic shock group with twelve mice in each phase. Blood pressure (BP) was monitored continuously by attaching carotid artery catheter to a strain gauge pressure transducer/ polygraph. Arterial blood samples were taken for blood gas analysis. A mouse model of non-lethal hemorrhagic shock and resuscitation was used to observe pulmonary myeloperoxidase (MPO) activity and wet/dry weight ratio (W/D). The expression of HO-1 was observed by means of RT-PCR and immunohistochemistry. IL-6 and IL-10 in lung tissue homogenate were assayed by enzyme-linked immunosorbent assay (ELISA). The pulmonary pathologic changes were observed under electron microscope and light microscope.

Results: Compared with sham group, the expression of HO-1 in lung tissue was significantly higher in Hem 24 h and Hem 48 h of C3H/HeN mice (P<0.01). The expression of HO-1 mRNA and the levels of IL-6, IL-10 and MPO in lung tissue were markedly increased in Hem 24 h (P<0.01 or P<0.05); Compared with C3H/HeN mice, the expression of HO-1 mRNA and the levels of IL-6 and IL-10 in C3H/HeJ mice significantly decreased in Hem 24 h and Hem 48 h (P<0.01 or P<0.05), and the W/D, MPO in C3H/HeJ mice were obviously lower in Hem 24 h (P<0.05). The injuries of lung tissues after hemorrhagic shock have been demonstrated by histological examination with electron microscope and light microscope.

Conclusions: TLR-4 and HO-1 might modulate the balance of pro- and anti-inflammatory processes in inflammatory reaction of hemorrhagic shock-induced ALI, and the activation of Toll-like receptor might induce the transcription activity of HO-1, which may play a key role in acute lung injury.

Key words: Shock, hemorrhagic; Toll-like receptor 4; Heme oxygenase-1; Interleukin-6; Interleukin-10
METHODS

Materials

Forty-eight male mice, including C3H/HeN mice (wild-type mice, endotoxin-sensitive), at the age of 6-8 weeks, were obtained from Beijing Laboratory Animal Research Center; and C3H/HeJ mice (containing a point mutation of TLR-4, endotoxin-tolerant), at the age of 6-8 weeks, were obtained from Shanghai Institute for Biological Sciences. The reagents and instruments were as follows: HO-1 polyclonal antibody (StressGen Biotechnologies, Victoria, Canada); SP kit (Zymed, CA, USA); mouse IL-6 and IL-10 ELISA kit (R & D Systems, Minneapolis, MN, USA); phosphate buffered saline (Sigma, USA); Trizole reagent (Invitrogen, Paisley, UK); RT-PCR kit (Promega, USA); MPO kit (Nanjing Jiancheng Co, China); high resolution pathological image analysis system (Tongji Technology Co, China); blood gas analyzer (i-STAT, USA); pressure transducer (Abbott, USA); V24E multiparameter monitor V24E (Philips).

Animal grouping and experimental processes

All the animals were kept on a 12-h /12-h light/dark cycle with free access to food and water, and acclimated for at least 7-10 days before experiments. We established a mouse model of non-lethal hemorrhagic shock and resuscitation, which Ayala and Fan had described.4,5 Mice were anaesthetized with an intraperitoneal injection of 4% chloral hydrate, and restrained in a supine position. The mice were sterilized as routine to expose the trachea, which were catheterized and mechanically ventilated with a fiction of 21% inspired oxygen (FiO₂). Carotid artery, femoral artery and caudal vena were catheterized under aseptic conditions with polyethylene 10# tube. The carotid arterial catheter was connected to a pressure transducer for the determination of continuous mean arterial blood pressure (MABP), while the femoral artery catheter was used for bleeding and induction of hemorrhagic shock. Hemorrhagic shock was initiated by blood withdrawal and reduction of the MABP to (40±5) mm Hg within 15 min (pre-hemorrhagic BP of 95 mm Hg). Blood was collected into a 1-ml syringe and heparinized to prevent clotting. After a hypotensive period of 60 minutes, animals were resuscitated by transfusion of the shed blood and Ringer’s lactate in a volume equal to that of the shed blood within 30 minutes. The catheters were then removed, the vessels ligated, and the groin incisions closed. The blood sample was assayed immediately after being taken from the carotid artery at 6, 24 and 48 hours after hemorrhagic shock. The mice were divided into sham group with six mice, hemorrhagic shock with six mice in each phase (Hem 6 h, 24 h, 48 h). Sham animals underwent the same surgical procedures without hemorrhage and resuscitation. The diagnostic evidence as hemorrhage induced ALI is PaO₂/FiO₂<300 mm Hg.

Polymerase chain reaction for HO-1 mRNA

Total cellular RNA was isolated from the lung of each mouse using Trizol reagent according to the protocol from manufacturers. HO-1 mRNA expression was analyzed by reverse transcriptase polymerase chain reaction (RT-PCR). β-actin was used as the gene transcript control. Primers used for β-actin were 5’ GCC TTC CTT GGG TAT 3’ and 3’ TTT GAC CTT GCC ACT TCC 5’. Primers used for HO-1 were 5’ TCACTGGCAGGAAATCAT3’ and 3’ CACTGTCTTCTCCGATTCTG5’, pre-denaturation at 95°C for 3 minutes, HO-1 cDNA for 32 cycles at 95°C (denaturation) for 30 seconds, 56°C (annealing) for 1 minute and 72°C (primer extension) for 90 seconds, followed at 72°C for 7 minutes. Densitometric analysis for semi-quantitation of PCR products was performed with an alpha imager 2000 analyzer.

Assessment of HO-1 expression of immunohistochemical staining

The lower lobe of right lung was taken out, fixed with 4% buffered paraformaldehyde and embedded in paraffin as routine. It was cut into sections with the thickness of 3 µm and stained with SP immunohistochemistry method. Randomly take several sections and use PBS instead of HO-1 polyclonal antibody as negative control (other procedures unchanged). The positively-stained sections were analyzed with automatic image analysis system. The mean value was calculated according to the results of random detection in five high power fields.

Cytokine enzyme-linked immunosorbent assay (ELISA)

IL-6 and IL-10 levels in lung tissue homogenates were determined by the “sandwich ELISA” technique. The procedure was based on the instruction for the variance or q test with SPSS 11.5 statistical software.
package. $P < 0.05$ was considered significantly different.

**RESULTS**

HO-1 mRNA expression in lung

The RT-PCR study revealed that the expression of HO-1 mRNA rapidly increased after hemorrhagic shock, peaking at 24 hours, followed by a gradual decrease. Compared with sham group, the expression of HO-1 mRNA of C3H/HeN mice were markedly increased in Hem 24 h group ($P=0.000$, $t=8.247$). Compared with C3H/HeN mice, the expressions of HO-1 mRNA of C3H/HeJ mice significantly decreased in Hem 24 h and Hem 48 h group ($P=0.009$, $t=3.261$ and $P=0.037$, $t=2.403$, Fig. 1).

**Immunohistochemical study of hemorrhagic shock induced ALI**

Immunohistochemical analysis showed a brownish cytoplasm for the cells in which HO-1 was positively expressed. Compared with sham group, the expression of HO-1 in lung tissue was significantly higher in Hem 24 h group and Hem 48 h group ($P=0.000$, $t=6.994$ and $P=0.014$, $t=2.970$). HO-1 was upregulated in the pulmonary endothelial cells, part of pulmonary vascular smooth muscle cells, extima of vessels and epithelial cells of airway. Compared with C3H/HeN mice, the expression of HO-1 of C3H/HeJ mice significantly decreased in Hem 24 h group ($P=0.000$, $t=5.534$, Table 2).

**IL-6 and IL-10 activity in the lung homogenates**

The levels of IL-6, IL-10 in C3H/HeN mice were significantly higher in Hem 24 h group than those of sham group ($P=0.000$, $t=14.810$ and $P=0.003$, $t=3.941$). Compared with C3H/HeN mice, the levels of IL-6 and IL-10 in C3H/HeJ mice significantly decreased in Hem 24 h group ($P=0.03$, $t=2.537$ and $P=0.037$, $t=2.400$, Table 3).

**W/D and MPO activity in the lung homogenates**

The change in the ratio of wet weight to dry weight was taken as an indicator of organ edema, and MPO activity represent an index of PMN tissue infiltration. Compared with sham group, W/D in lung tissue was highest in Hem 6 h group ($P=0.000$, $t=7.409$), and MPO were markedly increased in Hem 24 h and Hem 48 h group. Compared with C3H/HeN mice, W/D and MPO in C3H/HeJ mice were obviously lower in Hem 24 h group ($P=0.038$, $t=2.384$ and $P=0.049$, $t=2.239$, Table 4).

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**Table 1.** Changes of HO-1/β-actin expression in lung tissues ($n=6$, $\bar{x} \pm s$)

<table>
<thead>
<tr>
<th>Groups</th>
<th>HO-1 mRNA C3H/HeN</th>
<th>C3H/HeJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.80±0.234</td>
<td>0.77±0.241</td>
</tr>
<tr>
<td>Hem 6 h</td>
<td>1.17±0.283</td>
<td>1.16±0.259</td>
</tr>
<tr>
<td>Hem 24 h</td>
<td>2.19±0.341</td>
<td>1.62±0.266**</td>
</tr>
<tr>
<td>Hem 48 h</td>
<td>1.55±0.298**</td>
<td>1.15±0.276</td>
</tr>
</tbody>
</table>

**Table 2.** The expression of HO-1 in lung tissues (OD) ($n=6$, $\bar{x} \pm s$)

<table>
<thead>
<tr>
<th>Groups</th>
<th>HO-1 mRNA C3H/HeN</th>
<th>C3H/HeJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.034±0.010</td>
<td>0.036±0.008</td>
</tr>
<tr>
<td>Hem 6 h</td>
<td>0.047±0.011</td>
<td>0.045±0.010</td>
</tr>
<tr>
<td>Hem 24 h</td>
<td>0.152±0.040**</td>
<td>0.056±0.014**</td>
</tr>
<tr>
<td>Hem 48 h</td>
<td>0.054±0.013**</td>
<td>0.051±0.016</td>
</tr>
</tbody>
</table>

**Table 3.** IL-6 and IL-10 activity in lung homogenates ($n=6$, $\bar{x} \pm s$)

<table>
<thead>
<tr>
<th>Groups</th>
<th>IL-6 (pg/mg prot) C3H/HeN</th>
<th>C3H/HeJ</th>
<th>IL-10 (pg/mg prot) C3H/HeN</th>
<th>C3H/HeJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>60.74±14.47</td>
<td>55.62±12.83</td>
<td>95.91±20.54</td>
<td>80.79±24.30</td>
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<tr>
<td>Hem 6 h</td>
<td>72.23±19.06</td>
<td>68.52±11.97</td>
<td>100.35±22.32</td>
<td>95.23±19.54</td>
</tr>
<tr>
<td>Hem 24 h</td>
<td>365.38±48.26**</td>
<td>300.89±39.34**</td>
<td>161.49±35.21**</td>
<td>116.02±30.23**</td>
</tr>
<tr>
<td>Hem 48 h</td>
<td>297.85±27.41**</td>
<td>231.29±31.30**</td>
<td>132.40±14.76**</td>
<td>98.17±26.82</td>
</tr>
</tbody>
</table>

$^* P<0.05$, $^** P<0.01$, compared with sham group; $^* P<0.05$, compared with C3H/HeN group.
Alterations under electron microscope and light microscope

Under transmission electron microscope, at 6 hours and 48 hours post-hemorrhagic shock of C3H/HeN mice, we could observe the basement membranes of alveolar epithelium and capillary endothelium became swollen, widened, and their electronic density reduced, even disappeared. In Hem 24 h group, we could see the mitochondria in type II alveolar cells swollen, osmiophilic lamellar bodies emptied, and cell ridge lodged and disappeared. Moreover, the conjunctions between the alveolar epithelial cells and the capillary endothelial cells were injured with gaps. Under light microscope, in Hem 6 h group of C3H/HeN mice, there were congestion, edema and a few granulocytes in the alveolar cavity; at 24 hours post-hemorrhagic shock of C3H/HeN mice, alveolar cavity became narrow and alveolar wall thickened. There were considerable inflammatory cell infiltration in the pulmonary interstitium, and lymphocyte infiltration could be seen in a small number of alveoli and vascular vessels. In Hem 48 h group, the extent of damage was slighter than that in Hem 24 h group. Under both electron microscope and light microscope, compared with C3H/HeN mice, the pathologic changes of C3H/HeJ were significantly milder (Fig. 2).

**DISCUSSION**

Recent investigation has established TLR-4 as a major receptor for LPS-induced infectious responses. The results of previous studies on changes in innate immune responses after injury have shown that innate immune cell types such as neutrophils, blood monocytes and tissue macrophages display a heightened level of reactivity to microbial stimuli resulting in higher production of inflammatory mediators. Although increased innate immune reactivity may have been evolved to protect the injured host from developing opportunistic infections by boosting immune surveillance and cellular microbicidal activity, a high inflammatory response potential can also cause strong and possibly fatal systemic inflammation.

Consistent with our study, other researches have
shown that injury primes innate immune cells for stimulated proinflammatory cytokine production. It seemed that this resulted from increased TLR-4 responsiveness. Thus, we were interested in determining whether changes in TLR-4 responses could contribute to the development of severe ALI and SIRS. In this study, we found that irrespective of endotoxin-sensitive C3H/HeN or containing a point mutation of TLR-4 of C3H/HeJ in Hem 24 h group, a marked increase in lung IL-6, IL-10 and MPO levels were still observed as compared with sham mice. It indicates that all these animals are susceptible to hemorrhage-induced priming. However, at 6 hours after hemorrhagic shock, the extent of local inflammation observed was divergent in these animals. Compared with C3H/HeN, deficiency of TLR-4 gene product in the C3H/HeJ markedly reduced W/D and alveolar collapse, but did not alter the extent of local cytokine/chemokine expression. This reveals that at 6 hours after hemorrhagic shock, signaling through TLR-4 did not remarkably affect priming for the cytokine response to ALI. But at 24 hours after hemorrhagic shock, signaling through TLR-4 lead to priming for the cytokine response, the observation made here of increased lung cytokine/chemokine levels and the MPO activities in the C3H/HeJ mice imply that there is a potential anti-inflammatory pathway in these animals. We speculate that it is due to activated macrophages, neutrophils, or other immune and possible non-immune cells contributing to the release of these cytokines and neutrophil influx. Further study would be needed.

The systemic inflammatory response syndrome initiated by infection shares many features in common with the trauma-induced systemic response. The TLRs stand at the interface of innate immune activation in the settings of both infection and sterile injury by responding to a variety of microbial and endogenous ligands alike. Recently, the literature has described a key role for TLRs in acute injury using rodent models of hemorrhagic shock, ischemia and reperfusion, tissue trauma and wound repair. The very recent realization that certain TLR family members also respond to endogenous molecules released from stressed or damaged tissues points to a molecular basis for a shared mechanism of innate immune activation by infection and injury.

It was found that the body can secondarily produce heat shock proteins (HSPs) under the stress states such as hypoxia, injury and LPS attack. Shen et al found that beneficial effects following TLR-4 blockade were dependent on intrahepatic overexpression of HO-1 mRNA/protein. Hepatic IRI represents a case for innate immunity in which HO-1 modulates proinflammatory responses that are triggered via TLR-4 signaling, a putative HO-1 repressor. Zhan et al suggested that HO-1 enable to alleviate the pathological changes of ALI and it is an important stress-protection mechanism against endotoxic ALI. In high HO-1 expressed transgeneic rats, proinflammatory cytokine content substantially decreased, hypoxia-induced pulmonary inflammatory reaction, pulmonary hypertension, vascular wall proliferation were inhibited, and the pulmonary tissues were more tolerable to hypoxemia. In this study, we demonstrated that HO-1 provided significant protection from hemorrhage-induced ALI in Hem 24 h mice, and the expressions of HO-1 in lung tissue was highest in Hem 24 h group. At 24 hours after hemorrhage-induced ALI, we found that increased levels of HO-1 mRNA showed increased activation of both pro- and anti-inflammatory signals. However, anti-inflammatory mechanisms provided by HO-1 cannot keep pro-inflammatory activation in balance after hemorrhage. Compared with C3H/HeN mice, the expression of HO-1 mRNA of C3H/HeJ significantly decreased at 24 hours and 48 hours after hemorrhagic shock. It suggests that the activation of TLRs might induce the transcription activity of HO-1 or HO-1 modulated proinflammatory responses triggered via TLR-4 signaling.

REFERENCES

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