Toll-Like Receptor 4 Mediates Lung Ischemia-Reperfusion Injury

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Background. **We have previously reported that nuclear factor (NF)-κB activation and inflammatory cytokine expression were involved in the development of lung ischemia-reperfusion injury (LIRI). Because Toll-like receptor 4 (TLR4) activates NF-κB-dependent transcription of inflammatory cytokine genes during myocardial ischemia-reperfusion injury, we examined whether absence of TLR4 in TLR4-deficient mice protects against LIRI.**

Methods. **Left lungs of wild-type (C57BL/6J) mice or TLR4-null ($TLR4^{-/-}$) mice were made ischemic for 60 minutes and then reperfused for 180 minutes. Response to injury was quantified by tissue myeloperoxidase activity, vascular permeability ($[^{125}I]$-bovine serum albumin extravasation), and leukocyte and inflammatory mediator accumulation in bronchoalveolar lavage expression. Lung homogenates were also analyzed for activation of mitogen-activated protein kinases and nuclear translocation of the transcription factors NF-κB and activator protein-1.**

Results. **After LIRI, lungs from $TLR4^{-/-}$ mice demonstrated a 52.4% reduction in vascular permeability ($p = 0.001$), a 52.6% reduction in lung myeloperoxidase activity ($p = 0.006$), and a marked reduction in bronchoalveolar lavage leukocyte accumulation when compared with lungs from wild-type mice. The $TLR4^{-/-}$ mice lungs, subjected to LIRI, also demonstrated marked reductions in amounts of several proinflammatory cytokines/chemokines in bronchoalveolar lavage samples. Phosporylation of c-Jun NH_2-terminal kinase, and activation of NF-κB and activator protein-1 were also significantly reduced in homogenates of lungs from $TLR4^{-/-}$ mice injured by ischemia and reperfusion ($p < 0.05$).**

Conclusions. **These data suggest that TLR4 plays a role in LIRI. Thus, TLR4 may be a potential therapeutic target to minimize ischemic-reperfusion–induced tissue damage and organ dysfunction.**

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Direct lung ischemia-reperfusion (IR) injury (LIRI) is a morbid complication of lung and heart–lung transplantation. It has been associated with more frequent episodes of acute cellular rejection [1], and predisposes lung recipients to an earlier onset of chronic rejection or obliterative bronchiolitis [2]. The development of LIRI is also associated with pulmonary thromboendarterectomy, open heart surgery, aortic surgery, severe hemorrhagic shock, and after resuscitation for circulatory arrest. Therefore, strategies aimed at amelioration of this form of acute lung dysfunction may have substantial effectiveness for patient care in general.

During IR, cell-surface inflammatory receptors of the innate immune system are stimulated, resulting in activation of intracellular signaling cascades, with subsequent upregulation of transcription of several proinflammatory mediators including cytokines, chemokines, and adhesion molecules, producing a robust inflammatory response [3]. Recently, we have focused on an innate immune receptor, Toll-like receptor 4 (TLR4), and the role that TLR4 may have in LIRI. Previous studies of TLR4 have focused on TLR4 activation during gram-negative sepsis, and activation of TLR4 by endotoxin. However, we [4] and others [5] have demonstrated a role for TLR4 in myocardial IR injury that does not involve interaction of TLR4 with an endotoxin ligand. In addition, a recent study has shown that TLR4 interacts with a protein ligand released from damaged hepatocytes to initiate an IR injury in the liver [6].

Two pathways of injury that may occur during ischemia and reperfusion of lung tissue are apoptosis, induced through activation of a transcriptional program controlled by nuclear factor (NF)-κB; and acute inflammation, promoted by activation of resident alveolar macrophages and expression of several proinflammatory cytokines and chemokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-8, and macrophage inflammatory protein (MIP)-2 [7]. We also have previously reported that TLR4 activates NF-κB-dependent transcription of inflammatory cytokine genes in myocardial IR injury [4, 8]. The TLR4-mediated injury appears to occur through activation of c-Jun NH_2-terminal kinase (JNK) and translocation of NF-κB.

To examine the function of TLR4 in LIRI, we used a model of in situ single-lung ischemia and reperfusion

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Abbreviations and Acronyms

AP-1 = activator protein-1
BAL = bronchoalveolar lavage
BSA = bovine serum albumin
cpm = counts per minute
ERK = extracellular signaling-regulated kinase
HMGB1 = high-mobility group box 1
IL = interleukin
IR = ischemia-reperfusion
JNK = c-Jun NH_2-terminal kinase
KC = keratinocyte chemoattractant
LIRI = lung ischemia-reperfusion injury
LPS = lipopolysaccharide
MAPK = mitogen-activated protein kinase
MCP-1 = monocyte chemoattractant protein-1
MIP = macrophage inflammatory protein
MPO = myeloperoxidase
NF-κB = nuclear factor-κB
TLR = Toll-like receptor
TNF-α = tumor necrosis factor-α
WT = wild-type

in TLR4 knockout mice. We hypothesize that absence of TLR4 will reduce LIRI similar to the attenuation of myocardial IR injury we have observed in TLR4-null mice.

Material and Methods

Animals and Experimental Design

Male C57BL/6J mice (wild-type [WT]; CLEA Japan, Tokyo, Japan) or TLR4-null ($TLR4^{-/-}$) mice (on the same genetic background as WT and each back-crossed with C57BL/6J mice at least eight times [9]), aged 7 to 14 weeks and weighing 20 to 25 g, were subjected to 60 minutes of warm in situ single-lung ischemia followed by 180 minutes of reperfusion by occlusion and release of the left hilum, modified as previously described [10]. Mice were intubated and placed under mechanical ventilation (tidal volume = 0.75 mL, respiratory rate = 120 breaths per minute) with an inspired oxygen content of 60% after undergoing general anesthesia with pentobarbital sodium (100 mg/kg, intrapercutaniously). After a left anterolateral thoracotomy through the fifth intercostal space, all animals were given 5 U heparin intrapercutaneously. Five minutes after heparin administration, the left pulmonary hilum, including the left main bronchus, artery, and vein, was occluded with a noncrushing microvascular clamp under the left lung in an inflated state. At the end of the 60-minute ischemic period, the clamp was removed and the lung was allowed to ventilate and reperfuse for as long as 180 minutes. Both $TLR4^{-/-}$ and WT mice underwent sham operations consisting of a thoracotomy without cross-clamping of the pulmonary hilum. All animals were maintained in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health and also with the "Guideline for Animal Experiments in Mie University Graduate School of Medicine."

Lung Vascular Permeability Index

To measure endothelial vascular injury secondary to ischemia and reperfusion, a lung permeability index was calculated. The ^{125}I radiolabeled bovine serum albumin ([^{125}I]-BSA; PerkinElmer Life and Analytical Sciences, Wellesley, Massachusetts), titrated to an activity of 8×10^5 counts per minute (cpm) per dose, was injected intravenously in a final volume of 50 μL of 1% BSA/phosphate-buffered saline solution 5 minutes before removal of the hilar clamp. At the end of the experiment, at 180 minutes of reperfusion, the radioactivity counts were quantitated in the left and right lung, and 1 mL blood was sampled from the inferior vena cava. The permeability index (PI) was calculated as:

$$PI = \text{left lung (cpm)}/1\ \text{mL blood (cpm)}$$

This ratio corrects for any variation in systemic blood levels of radioactivity and provides a reproducible indicator of lung microvascular permeability secondary to acute oxidative lung injury.

Bronchoalveolar Lavage

Recovered left lung bronchoalveolar lavage (BAL) effluent afforded analysis of inflammatory cell accumulation in the alveolar spaces of ischemia-reperfused lungs. Left lungs were lavaged selectively through the tracheostomy tube with 300 μL 0.9% saline after a clamp was placed on the right hilum. At least 80% of instilled volume needed to be recovered for the sample to be considered adequate. This fluid was centrifuged (1,500*g* for 8 minutes at 4°C) to pellet the cells. The supernatant was snap frozen for cytokine analysis, and then stored at −80°C until subsequent analysis. Inflammatory cells were then counted using a hemacytometer.

Analysis for Myeloperoxidase Activity

The myeloperoxidase (MPO) activity was used to quantitate lung parenchymal neutrophil accumulation. Frozen tissue samples were homogenized with ice-cold lysis buffer containing hexadecyltrimethylammonium bromide, and centrifuged, and supernatants were assayed for MPO activity using substrate buffer containing o-dianisidine dihydrochloride. The MPO activity was measured at an absorbance of 460 nm over 1 minute, and calculated as ΔOD_{460} nm/minute.

Western Immunoblotting Assay for Phosphorylation of Mitogen-Activated Protein Kinases

Whole-cell protein, extracted from frozen tissue samples with ice-cold lysis buffer (Cell Signaling Technology, Beverly, Massachusetts), were stored at −80°C until the time of assay. Whole-cell protein, 10 μg, was loaded onto 15% sodium dodecylsulfate polyacrylamide electrophoretic gels, and transferred to polyvinylidene difluoride membranes. The membranes were immunoblotted with primary antibodies contained in PhosphoPlus p38 mitogen-activated protein kinase (MAPK, Thr_{180}/Tyr_{182}), JNK (Thr_{183}/Tyr_{185}), and extracellular signal-regulated kinase (ERK, Thr_{202}/Tyr_{204}) Antibody Kits (1:1000; Cell