



Inhibition of Toll-like receptor 4 signaling ameliorates lung ischemia–reperfusion injury in acute hyperglycemic conditions

Mamoru Takahashi, MD, Toyofumi F. Chen-Yoshikawa, MD, PhD, Toshi Menju, MD, PhD, Keiji Ohata, MD, Takeshi Kondo, MD, Hideki Motoyama, MD, Kyoko Hijiya, MD, Akihiro Aoyama, MD, PhD, and Hiroshi Date, MD, PhD

From the Department of Thoracic Surgery, Kyoto University Graduate School of Medicine, Kyoto, Japan.

KEYWORDS:

lung ischemia–reperfusion injury;
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pro-inflammatory cytokines

BACKGROUND: Recent lung transplantation studies have shown that peri-operative hyperglycemia is an important factor affecting recipient survival; however, its underlying mechanisms are not well understood. We hypothesized that acute hyperglycemia exacerbates lung ischemia–reperfusion injury (IRI) through up-regulation of Toll-like receptor 4 (TLR4) signaling pathways.

METHODS: C57BL/6Ncr mice were divided into 3 treatment groups: sham; IRI; and IRI under acute hyperglycemic conditions (IRI+HG). Mice in the IRI and IRI+HG groups were exposed to IRI via clamping the left hilum for 1 hour, followed by reperfusion for 2 hours. Acute hyperglycemia was established by glucose injection. The severity of lung injury and TLR4 signaling pathway activity were assessed. Further, we performed a pharmacologic blockade of TLR4 signaling to determine the effect of TLR4 signaling inhibition on lung injury.

RESULTS: Compared with normoglycemic mice, hyperglycemic mice had 2-fold higher blood glucose levels ($p < 0.001$). Pulmonary compliance was significantly lower, and airway resistance was significantly higher, in the IRI+HG group than in the IRI group ($p < 0.05$). Levels of inflammatory cytokines in bronchoalveolar lavage fluid were significantly higher in the IRI+HG group than in the IRI group. Correspondingly, TLR4 signaling pathways were up-regulated in the IRI+HG group. Moreover, pharmacologic inhibition of TLR4 signaling significantly decreased lung injury markers under hyperglycemic conditions.

CONCLUSIONS: Acute hyperglycemia exacerbated lung IRI and was associated with up-regulation of TLR4 signaling pathways. Pharmacologic inhibition of TLR4 signaling ameliorated lung IRI with acute hyperglycemia. Targeting TLR4 appears to be a promising approach to managing coexisting pathologies in lung transplant recipients.

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Acute (stress-induced) hyperglycemia occurs during organ transplantation in known diabetic patients as well as in

non-diabetic transplant recipients.^{1,2} Several lung transplantation studies have suggested that peri-operative hyperglycemia is a key contributing factor to the poor survival of lung transplant recipients; however, the underlying mechanisms affecting patient survival have not been fully elucidated.^{3,4}

A recent study in mice showed that acute hyperglycemia exacerbates lung ischemia–reperfusion injury (IRI) through

Reprint requests: Hiroshi Date, MD, PhD, Department of Thoracic Surgery, Kyoto University Graduate School of Medicine, 54 Shogoin-kawahara-cho, Sakyo, Kyoto City, 606-8507, Japan. Telephone: +81-75-751-4975. Fax: +81-75-751-4974.
E-mail address: hdate@kuhp.kyoto-u.ac.jp

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the receptor for advanced glycation end-products (RAGEs), a group of non-enzymatically glycated byproducts.⁵ Lung IRI can result in acute graft failure, which is the leading cause of death in the early post-transplant period.^{6–8} In addition, lung IRI is one of the major causes of chronic graft rejection among patients undergoing lung transplantation.⁹ Therefore, studies aimed at exacerbating factors of IRI hold substantial therapeutic value.

Toll-like receptor 4 (TLR4) signaling is known to play a crucial role in lung IRI.¹⁰ Although TLR4 was originally identified as a pattern-recognition receptor that was activated by specific bacterial ligands such as lipopolysaccharide (LPS), it is now well known that TLR4 signaling is responsive to oxidative stress caused by a wide variety of acute lung injuries, including lung IRI.^{11,12} Interestingly, studies using cardiomyocytes and mesangial cells have shown that hyperglycemic conditions can activate TLR4 signaling through enhanced oxidative stress.^{13,14} Although it is unclear whether hyperglycemic conditions affect lung IRI through the TLR4 signaling pathway, exploring these mechanisms will help determine the importance of peri-operative glycemic control during lung transplantation.

In this study, we focused on acute hyperglycemia and hypothesize that acute hyperglycemia could exacerbate lung IRI through activation of TLR4 signaling pathways. We initially evaluated markers of lung IRI and TLR4 signaling activity under hyperglycemic conditions. Next, we evaluated whether inhibition of TLR4 signaling attenuated lung IRI injury under acute hyperglycemic conditions.

Methods

Study design and surgical model

All animal experiments were performed using animal protocols approved by the institutional animal care and use committee of Kyoto University. We used C57BL/6Ncr wild-type mice (Japan SLC, Hamamatsu, Japan), 15 to 17 weeks of age and weighing 26 to 28 g. Surgical procedures were performed as previously described, with several modifications.^{5,15} Briefly, mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (100 mg/kg). The tracheotomy allowed mechanical ventilation with a tidal volume of 0.75 ml, respiratory rate of 120 breaths/min, positive end-expiratory pressure of 3 cm H₂O and fraction of inspired oxygen (FIO₂) of 1.0. Then, 200 U/kg heparin sodium was injected subcutaneously. Ten minutes after the heparin injection, the left pulmonary hilum was occluded for 1 hour using a microvascular clamp through a left thoracotomy. Reperfusion began when the clamp was removed and was maintained for 2 hours. Mice were randomly assigned to the following 3 groups, with each group containing 5 or 6 mice: (1) a sham group (left thoracotomy + 2-hour perfusion); (2) a normoglycemic ischemia–reperfusion injury group (IRI group; 1-hour left lung ischemia + 2-hour reperfusion in the absence of acute hyperglycemia); and (3) a hyperglycemic ischemia–reperfusion injury group (IRI+HG group; 1-hour left lung ischemia + 2-hour reperfusion in the presence of acute hyperglycemia). Mice were euthanized by cardiectomy at the end of the reperfusion period. Acute hyperglycemia was established via intraperitoneal injection of 10% glucose solution (0.3 ml per

mouse) at 30 minutes before ischemia induction. We used normal saline (0.3 ml per mouse) as a negative control. The glucose dosing strategy was based on reports described elsewhere.^{5,16} Blood glucose levels were measured via the left jugular vein at the following 3 time-points: start of ischemia; start of reperfusion; and end of reperfusion.

Pulmonary function

At the end of reperfusion, we clamped the right hilum and measured left lung function (airway resistance and pulmonary compliance) using a flexiVent (SCIREQ, Montreal, Canada), according to the manufacturer's instructions.

Bronchoalveolar lavage

After measurement of pulmonary functions, bronchoalveolar lavage (BAL) of the left lung was performed with the right hilum clamped. Phosphate-buffered saline (PBS; 0.6 ml, pre-warmed at room temperature) was instilled in the trachea and returned fluid was centrifuged immediately (500 × g, 5 minutes, 4°C). Concentrations of intercellular adhesion molecule-1 (ICAM-1), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) in these undiluted BAL supernatants were measured using a commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kit, as specified by the manufacturers. We purchased the IL-6 and MCP-1 kits from BioLegend (San Diego, CA) and the ICAM-1 kit from RayBiotech, Inc. (Norcross, GA).

Western blot analysis

Protein concentration and Western blot analyses were performed as described elsewhere.¹⁵ Briefly, after collecting BAL fluids, the left lung was snap-frozen in liquid nitrogen and then stored at –80°C until needed for analysis. Frozen lung tissue was suspended in 10 µl/mg ice-cold radioimmunoprecipitation buffer (1% NP-40, 1% deoxycholate, 0.1% sodium dodecylsulfate, 20 mmol/liter Tris [pH 7.4], 150 mmol/liter NaCl, 5 mmol/liter ethylene-diamine tetraacetic acid NaCl, 1 mmol/liter phenylmethanesulfonyl [PMSF], 5 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml Pepstatin A, 1 mmol/liter vanadate). Protein concentrations were determined using the Bradford method after Dounce homogenization and centrifugation (15,000 × g, 10 minutes, 4°C). Protein (10 µg) was loaded onto a 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were incubated with the following primary antibodies to evaluate TLR4 expression and an upstream pathway: anti-TLR4 (LifeSpan BioSciences, Nottingham, UK) and anti-high-mobility group box 1 (HMGB1; Sigma-Aldrich Japan, Tokyo, Japan). The following primary antibodies were used to evaluate downstream pathways of TLR4: anti-p38; phosphorylated p38 (Sigma-Aldrich Japan, Tokyo, Japan); extracellular signal-regulated kinase 1/2 (ERK1/2); phosphorylated ERK1/2; Akt; and phosphorylated Akt antibodies (Cell Signaling Technology, Inc., Beverly, MA). Blots were developed using an electrochemiluminescence system (GE Healthcare, Buckinghamshire, UK) and visualized on an imager (LAS 3000; GE Healthcare Japan, Tokyo, Japan). Band densitometry analysis was performed using Image J 1.47 software (NIH, Bethesda, MD). β-actin expression was used as an internal control.

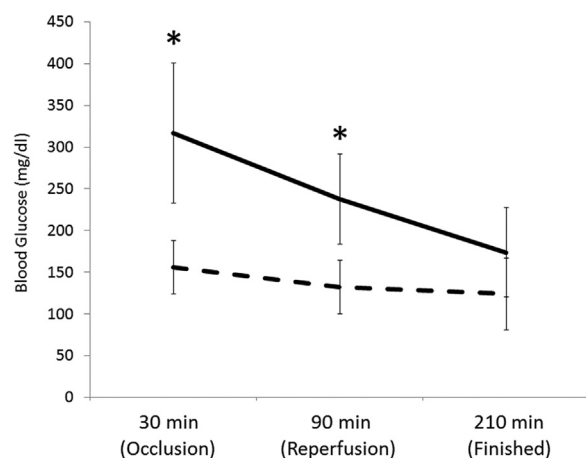


Figure 1 Time-course of blood glucose levels in glucose-treated mice (intraperitoneal injection of 10% glucose, 0.3 ml per mouse) and untreated mice (intraperitoneal injection of normal saline, 0.3 ml per mouse) after glucose injection (30 minutes before ischemia induction). The solid line indicates the time-course in treated mice (glucose). The dashed line indicates the time-course in untreated mice (normal saline). The ischemia and reperfusion periods begin at 30 and 90 minutes, respectively. The reperfusion period is complete at 210 minutes. * $p < 0.05$ treated mice vs untreated mice.

Pathologic evaluation

At the end of reperfusion, lung blocks were inflation-fixed through the trachea with 10% formalin for 24 hours at room temperature and then embedded in paraffin. Sections were stained with hematoxylin and eosin.

Pulmonary vascular permeability

Using a separate group of mice ($n = 5$ per group), vascular permeability of the left lung was evaluated based on the Evans blue dye (EBD) technique, as previously reported.¹⁵ Briefly, after occlusion of the left hilum, 30 mg/kg of EBD in 250 μ l of normal saline solution was injected into the left jugular vein. After 1 hour of occlusion and 2 hours of reperfusion, the left lung was flushed with normal saline (30 ml) to remove intravascular EBD. The left

tissue was suspended in formamide (100 mg lung tissue per 1 ml formamide) for 24 hours at 50°C. Specimens were centrifuged ($13,000 \times g$, 30 minutes), and 100 μ l supernatant was placed in 96-well plates for colorimetric analysis in a μ Quant spectrophotometer (Bio-Rad Laboratories K.K., Tokyo, Japan) at 620 nm. Relative optical density values were normalized to the weight of the samples.

TLR4 inhibition

VIPER, a TLR4 inhibitor, was purchased from Novus Biologicals (Littleton, CO). This TLR4-specific inhibitor blocks TLR4 signaling at the receptor adaptor level, down-regulating phosphorylation of mitogen-activated protein kinases (MAPK) and pro-inflammatory cytokine expression.¹⁷ In this experiment, mice were injected with the VIPER solution (1.0 mg/kg, 0.5 ml PBS solution) via the left jugular vein 15 minutes before inducing ischemia. The inhibitor dose was determined according to the manufacturer's instructions. We used the CP7 solution (1.0 mg/kg, 0.5 ml PBS solution) as a negative control, according to the manufacturer's instructions.

Statistical analysis

All values are presented as the mean $\pm$ standard deviation. Data were evaluated using one-way analysis of variance (ANOVA) to determine differences between groups. $p < 0.05$ was considered statistically significant.

Results

Time-course of blood glucose levels

The time-course of blood glucose levels is shown in Figure 1. Glucose levels before ischemia were 317 ± 84 mg/dl in the IRI+HG group and 156 ± 32 mg/dl in the IRI group ($p < 0.001$). The glucose levels before reperfusion were 238 ± 54 mg/dl in the IRI+HG group compared with 132 ± 32 mg/dl in the IRI group ($p < 0.001$). The glucose levels at the end of reperfusion were 174 ± 54 mg/dl in the IRI+HG group and 124 ± 43 mg/dl in the IRI group ($p = 0.16$).

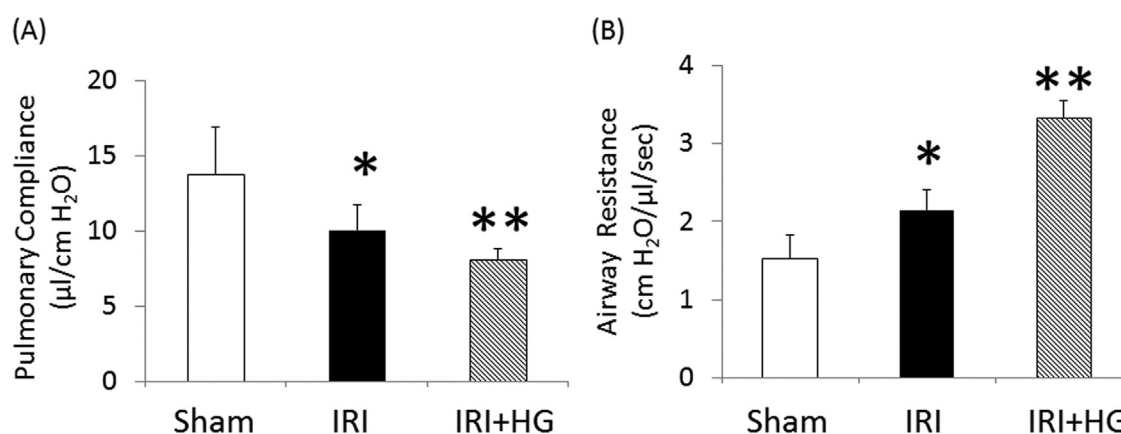


Figure 2 Left lung function after IRI. (A) Airway resistance and (B) pulmonary compliance. Sham, sham surgery; IRI, ischemia-reperfusion injury; IRI+HG, ischemia-reperfusion injury + acute hyperglycemia. * $p < 0.05$ vs sham; ** $p < 0.05$ vs IRI+HG.

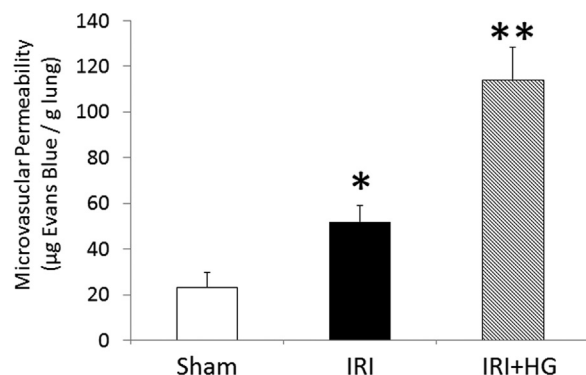


Figure 3 Vascular permeability quantified using Evans blue extravasation. Sham, sham surgery; IRI, ischemia–reperfusion injury; IRI+HG, ischemia–reperfusion injury + acute hyperglycemia. * $p < 0.05$ vs sham; ** $p < 0.05$ vs IR.

Effect of acute hyperglycemia on pulmonary function and pulmonary vascular permeability after IRI

The results of the lung function analysis are shown in Figure 2. Pulmonary compliance was lower and airway resistance was higher in the IRI+HG group than in the IRI group ($p < 0.05$). Histologic analysis revealed that more erythrocytes accumulated in the alveoli of the IRI+HG group

than the IRI group (not shown). As shown in Figure 3, pulmonary vascular permeability was 2-fold higher in the IRI group than in the sham group ($p < 0.05$) and, similarly, pulmonary vascular permeability in the IRI+HG group was higher than that in the IRI group ($p < 0.05$).

Effect of hyperglycemia on inflammatory cytokine production in bronchoalveolar lavage after IRI

Levels of ICAM-1, IL-6 and MCP-1 in BAL fluid were significantly increased in the IRI group compared with the sham group ($p < 0.05$) (Figure 4). Moreover, the levels of these cytokines in the IRI+HG group were significantly higher after IRI than those in the IRI group.

Effect of acute hyperglycemia on pulmonary TLR4 expression after IRI

Figure 5 demonstrates the results of Western blot analysis characterizing the TLR4 signaling pathway. TLR4 and HMGB1 bands in the IRI+ HG group were more pronounced than in the IRI group (Figure 5A and B). Similarly, phosphorylation of p38 and ERK1/2 was upregulated in the IRI+HG group (Figure 5C and D).

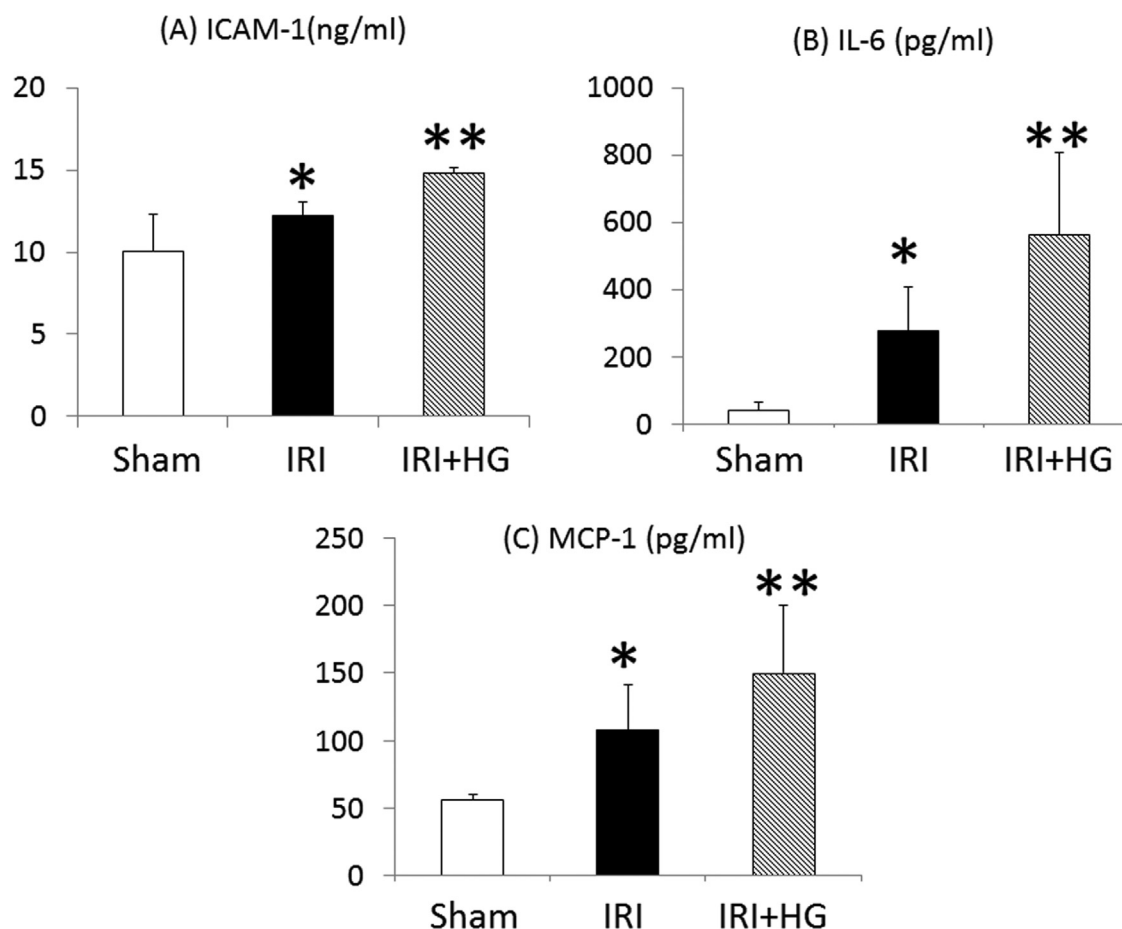


Figure 4 Measurement of inflammatory cytokine levels in BAL fluid in mice. (A) ICAM-1, (B) IL-6 and (C) MCP-1. ICAM-1, intercellular adhesion molecule-1; IL, interleukin; MCP-1, monocyte chemotactic protein-1; BAL, bronchoalveolar lavage; Sham, sham surgery; IRI, ischemia–reperfusion injury; IRI+HG, ischemia–reperfusion injury + acute hyperglycemia. * $p < 0.05$ vs sham; ** $p < 0.05$ vs IR.

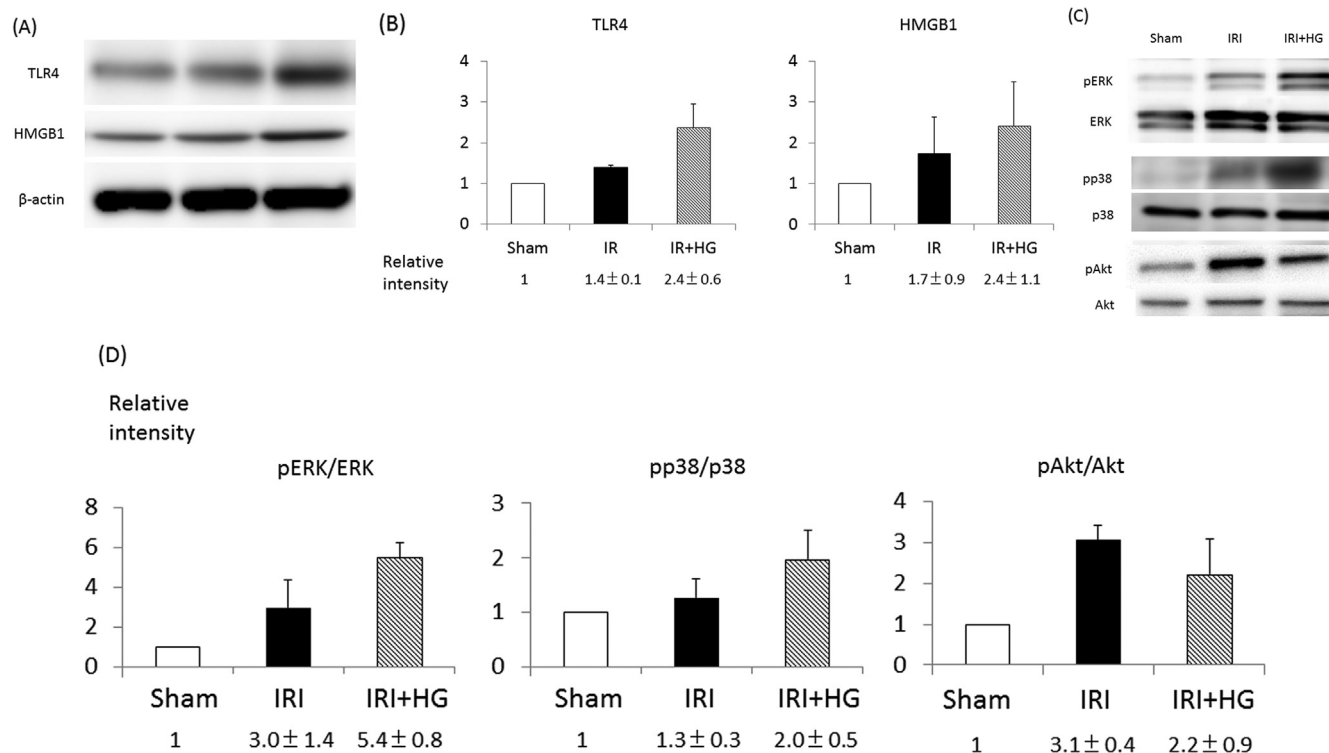


Figure 5 Western blotting analysis of TLR4 signaling pathways during lung IRI. (A) Representative images for TLR4 and HMGB1 in the left lung. β -actin served as an internal control. (B) Quantification of the expression of TLR4 and HMGB1 ($n = 4$ per group). (C) Representative images for phosphorylation of ERK1/2, p38 and Akt. (D) Quantification of phosphorylation of ERK1/2, p38 and Akt ($n = 4$ per group). TLR4, Toll-like receptor 4; HMGB1, high-mobility group box 1; ERK, extracellular signal-regulated kinase; Sham, sham surgery; IRI, ischemia-reperfusion injury; IRI+HG, ischemia-reperfusion injury + acute hyperglycemia.

Conversely, phosphorylation of Akt was not up-regulated in the IRI+HG group.

Physiologic changes by pharmacologic TLR4 inhibition during IRI

We evaluated the pharmacologic effect of VIPER on TLR4 signaling by Western blot analysis of ERK1/2. As shown in Figure 6, phosphorylation of ERK1/2 was suppressed in mice treated with this TLR4 inhibitor, whereas treatment with the inhibitor did not alter the expression of TLR4. We also confirmed that this pharmacologic intervention did not affect blood glucose levels in mice (data not shown). As shown in Figure 7, vascular permeability in the left lungs and cytokine levels (ICAM-1, IL-6 and MCP-1) in the BAL fluid were significantly lower in normoglycemic mice treated with VIPER compared with CP7-treated mice. Moreover, treatment with VIPER resulted in a significant decrease in vascular permeability and levels of these cytokines in BAL fluid in hyperglycemic as compared with CP7-treated mice.

Discussion

This study is the first to show that acute hyperglycemia significantly exacerbates lung IRI through TLR4 signaling. More importantly, we found that pharmacologic inhibition of TLR4 signaling could ameliorate lung IRI in acute hyperglycemic conditions.

The role of peri-operative hyperglycemia during organ transplantation is important in both diabetic and non-diabetic patients because organ transplant recipients have risk factors that induce high blood glucose levels. For instance, acute hyperglycemia can occur via ischemic insults to lung allografts as well as via biologic responses to

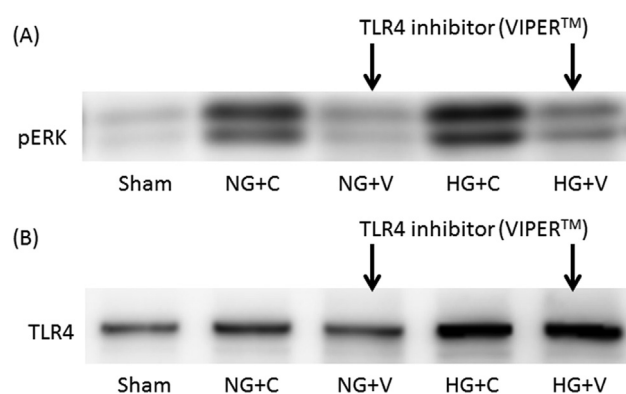


Figure 6 Western blot analysis of effects of the specific TLR4 inhibitor (VIPER) during lung IRI. (A) ERK phosphorylation is down-regulated in the presence of VIPER both in the absence (NG+V) and presence (HG+V) of acute hyperglycemia compared with that in negative controls (NG+C and HG+C). (B) The expression of TLR4 is not affected by inhibitory agents under normoglycemia and hyperglycemia. TLR4, Toll-like receptor 4; ERK, extracellular signal-regulated kinase; IRI, ischemia-reperfusion injury; V, VIPER; C, CP7; Sham, sham surgery; NG, ischemia-reperfusion injury + normoglycemia; HG, ischemia-reperfusion injury + acute hyperglycemia.

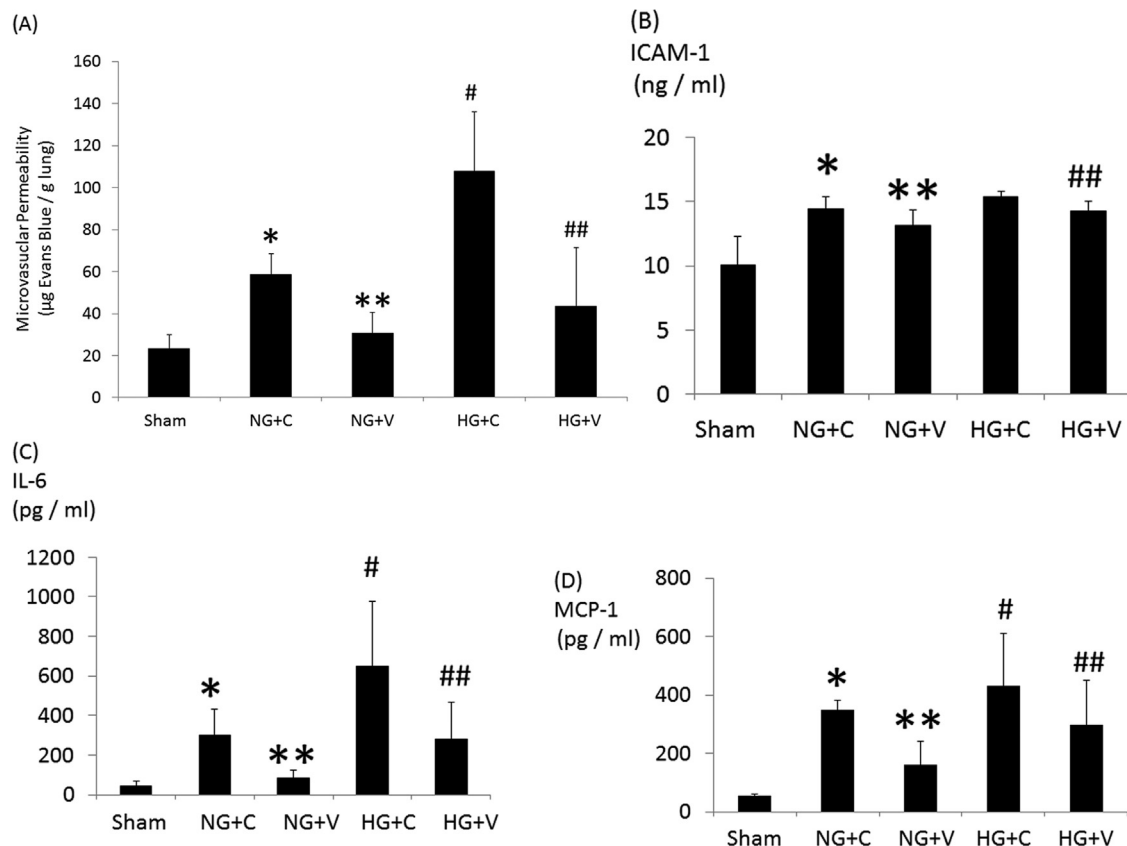


Figure 7 Pharmacologic inhibition of TLR4 signaling during lung IRI in the presence and absence of acute hyperglycemia. (A) Pharmacologic inhibition of TLR4 signaling reduces vascular permeability after lung IRI both in the presence (HG+V) and absence (NG+V) of acute hyperglycemia compared with negative control groups (NG+C and HG+C). (B–D) Levels of inflammatory cytokines in BAL fluid are reduced because of pharmacologic inhibition both in the presence (HG+V) and absence (NG+V) of acute hyperglycemia as compared with those in the negative control groups (NG+C and HG+C). (B) ICAM-1, (C) IL-6 and (D) MCP-1. * $p < 0.05$ vs sham; ** $p < 0.05$ vs NG; # $p < 0.05$ vs HG. TLR4, Toll-like receptor 4; IRI, ischemia-reperfusion injury; V, VIPER; C, CP7; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; BAL, bronchoalveolar lavage; Sham, sham surgery; NG, ischemia-reperfusion injury + normoglycemia; HG, ischemia-reperfusion injury + acute hyperglycemia.

surgical procedures.^{1,2} In addition, steroids or calcineurin inhibitors administered to prevent allograft rejection can induce acute and chronic hyperglycemia.^{8,18} In fact, post-transplant diabetes mellitus is found in almost 25% of lung transplant recipients.^{3,8} Although recent studies have suggested that diabetes is a major risk factor for morbidity and mortality in lung transplant recipients, the mechanisms underlying the effects of acute and chronic hyperglycemia on lung allografts are not fully understood.^{3,4}

In this study, we first confirmed that acute hyperglycemia significantly exacerbates lung IRI. The hyperglycemic mice in this study had 2-fold higher blood glucose levels than control mice, which is similar to stress-induced changes in transplant patients.^{2,5} We found that acute hyperglycemia significantly worsened physiologic parameters (airway resistance, pulmonary compliance and vascular permeability) after the ischemia-reperfusion period. We also observed elevated inflammatory cytokine production (ICAM-1, IL-6 and MCP-1) in BAL fluids, which closely reflects the severity of local lung injury.¹⁰ These pro-inflammatory effects of acute hyperglycemia were consistent with those in previous reports examining several animal models, including a mouse model of lung IRI.^{5,18}

We next investigated the underlying signaling pathways in which acute hyperglycemia exacerbates lung IRI, focusing on TLR4. Previous studies have shown that TLR4 signaling critically modulates lung IRI by recognizing endogenous molecules released from injured or dead cells.^{10,15} Furthermore, clinical studies have indicated that TLR4 expression correlates with pro-inflammatory cytokine production and lung allograft rejection.⁶

In our study, TLR4 expression in the injured lung was significantly higher in hyperglycemic conditions than in normoglycemia after IRI. In addition, the amount of HMGB1, an endogenously derived ligand of TLR4, in the injured lung was greater under hyperglycemic conditions.¹⁹ Hagiwara et al reported that hyperglycemia up-regulates the production of HMGB1 in LPS-induced acute lung injury.¹⁸ We suggested that a similar phenomenon occurred in the lung IRI model described in this study. Moreover, phosphorylation of MAPKs (ERK1/2 and p38) was up-regulated after lung IRI under acute hyperglycemic conditions. These findings are consistent with those of previous studies showing that activation of MAPKs, such as p38 and ERK1/2, corresponds to the severity of lung IRI.^{20,21} In contrast to MAPKs, phosphorylation of Akt was

not up-regulated in response to elevated blood glucose levels. Akt exerts cytoprotective effects through regulation of stress-related kinases, such as JNK and p38.^{22,23} Although this finding was not decisive, it may be indirect evidence that MAPKs were up-regulated. Thus, our results demonstrate that hyperglycemia can up-regulate HMGB1 during lung IRI, which, in turn, activates TLR4–MAPK interactions, leading to severe lung IRI.

Last, we have shown that pharmacologic inhibition of TLR4 signaling ameliorated lung IRI in acute hyperglycemic conditions. Several animal studies have revealed that genetic or pharmacologic inhibition of TLR4 attenuated the severity of lung IRI in the normoglycemic state.^{11,15} However, no previous study has demonstrated whether blockade of TLR4 could attenuate lung IRI under hyperglycemic conditions. In our study, TLR4 inhibition decreased vascular permeability and inflammatory cytokine production (ICAM-1, IL-6 and MCP-1) in the injured lung under both normoglycemic and hyperglycemic conditions. Our results suggest that pharmacologic inhibition of TLR4 could attenuate lung IRI even under hyperglycemic conditions.

Although our investigation focused on peri-operative acute hyperglycemia as a coexisting factor in lung transplantation, the potential protective effect of TLR4 inhibition may extend to other complications in lung transplant recipients. For example, lung transplant recipients often require pharmacologic treatments for pulmonary hypertension, hyperlipidemia and hyperglycemic conditions, and TLR4 has a vital role in the inflammatory process of endothelial dysfunction caused by these pathologies.^{8,24–26} Thus, targeting TLR4 could be an additional therapeutic option when these complications are refractory to conventional treatments. Further research is needed to determine whether targeting TLR4 signaling effectively reduces lung IRI and coexisting pathologies in lung transplant recipients.

There are several limitations to our study. First, we used a pharmacologic agent to block TLR4 signaling. Although our experimental settings suggest therapeutic efficacy of pharmacologic inhibition of TLR4, studies in TLR4 knock-out animal models are warranted to confirm the precise relationship between acute hyperglycemia and TLR4 signaling. Second, the role of TLR4 during acute hyperglycemia after lung IRI may not be generalized to a patient in a chronic hyperglycemic state. Although acute hyperglycemia and diabetes-associated hyperglycemia share many properties, the majority of diabetic patients have additional features of metabolic syndrome, such as insulin resistance, obesity, and dyslipidemia.¹⁶ Third, our study could not identify the precise cellular site for TLR4 activation during lung IRI. Recent reports have shown that alveolar macrophages play a crucial role in lung IRI.^{27,28} In our study, the severity of lung IRI correlated positively with expression of ERK1/2, which localizes to epithelial cells but not alveolar macrophages.²⁰ Thus, our results suggest that hyperglycemic conditions promote lung IRI via TLR4, at least in pulmonary parenchymal cells. Identifying the precise cellular sites of TLR4 activation is needed to fully understand lung IRI and further studies are warranted.

In conclusion, we found that acute hyperglycemia significantly exacerbated lung IRI through TLR4 signaling. Targeting TLR4 appears to be a promising approach for managing coexisting pathologies in lung transplant recipients.

Disclosure statement

The authors have no conflicts of interest to disclose.

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