Ischemic preconditioning attenuates acute lung injury after partial liver transplantation

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Abstract: Pulmonary complications frequently occur after liver transplantation and are often life-threatening. Thus, we investigated whether hepatic ischemic preconditioning (IP) attenuates acute lung injury (ALI) after small-for-size liver transplantation. Rat livers were explanted after 9-min ischemia plus 5-min reperfusion, reduced to 50% of original size ex vivo, and implanted into recipients with approximately twice the donor body weight, resulting in quarter-size liver grafts (QSG). After QSG transplantation, hepatic Toll-like receptor 4 (TLR4) and tumor necrosis factor-α (TNFα) expression increased markedly and high mobility group box-1 (HMGB1), an endogenous damage-associated molecular pattern molecule (DAMP), was released from QSG into the blood. IP blunted TLR4 and TNFα expression and HMGB1 release from QSG. In the lungs of QSG recipients without IP treatment, nuclear factor-κB (NF-κB) activation and intercellular adhesion molecule (ICAM)-1 expression increased; alveolar septal walls thickened with increased cellularity as neutrophils, monocytes/macrophage and T lymphocytes infiltrated into alveolar septa and alveolar spaces; and pulmonary cleaved caspase-8 and -3 and TUNEL-positive cells increased. In contrast, in the lungs of recipients of ischemic-preconditioned QSG, NF-κB activation and ICAM-1 expression were blunted; leukocyte infiltration was decreased; and alveolar septal wall thickening, caspase activation, and cell apoptosis were attenuated. IP did not increase heat-shock proteins in the lungs of QSG recipients. In conclusion, toxic cytokine and HMGB1 released from failing small-for-size grafts leads to pulmonary adhesion molecule expression, leukocyte infiltration and injury. IP prevents DAMP release and toxic cytokine formation in small-for-size grafts, thereby attenuating ALI.

Keywords: Acute lung injury, damage-associated molecular pattern molecule, heat shock protein, inflammation, ischemic preconditioning, partial liver transplantation

Introduction

Orthotopic liver transplantation remains the only proven therapy for end-stage liver disease [1, 2]. However, pulmonary complications occurring after liver transplantation are often life-threatening [3-7], and some reports indicate that the mortality for acute respiratory distress syndrome exceeds 50% [3, 4, 8]. Extended cold storage, ischemia/reperfusion-induced graft injury, and transfusion of plasma-containing blood products during surgery are documented to increase the occurrence of pulmonary complications [3, 4, 7-11]. Recently, partial liver transplantation has been increasingly utilized to alleviate the severe shortage of donor organs [12-14]. However, small-for-size syndrome occurs after transplantation of partial liver grafts with a relative volume less than 30-40% of the standard recipient liver volume, which leads to slow/no recovery of liver function, more severe post-transplantation complications, and increased mortality [12, 15]. Patients with acute hepatic failure often have pulmonary complications [16]. Moreover, trauma during liver resection/splitting procedures of partial liver transplantation stimulates inflammation responses in the lung [17], and hemodynamic alterations that occur after partial liver transplantation may exacerbate pulmonary complications [18, 19]. Thus, cross-talk between the liver and lung may exist, contributing to increased pulmonary complications after small-for-size liver transplantation. Indeed, our recent work indicates that pulmonary inflammation and injury increased after small-for-size liver transplantation in rats [20]. Because liver
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Table 1. Sources of reagents

<table>
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<th>Reagents</th>
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<td>Chemiluminescence kit</td>
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<td>UW cold storage solution</td>
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HMGB1, high mobility group box-1; ICAM-1, intercellular adhesion molecule-1; MPO, myeloperoxidase; NF-κB, nuclear factor-κB; TLR4, Toll-like receptor-4; TNFα, tumor necrosis factor-α. UW, University of Wisconsin.

Sources for reagents are listed in Table 1.

Ischemic preconditioning and liver transplantation

IP and liver transplantation were performed in male Lewis rats (170-200 g, Charles River, Durham, NC), as described previously [35-37]. Briefly, the donor abdomen was opened under ether anesthesia, and heparin (200 U) was injected into the vena cava. IP was induced 2 min later by clamping the portal vein and hepatice artery for 9 min. Livers were flushed in situ with 5 ml UW cold storage solution (0-1°C) via the portal vein 5 min after releasing the vascular clamp, and then livers were explanted. In ice-cold UW solution, liver mass was reduced ex vivo to ~50% of original size, as described [35-37], and unreduced and reduced-size livers were stored in UW solution at 0-1°C for 6 h. Reduced-size liver explants were implanted into recipients of greater body weight (350-420 g), which results in a graft weight/standard liver weight of ~25% (quarter-size graft, QSG), as described [35, 36]. Unreduced livers were implanted into recipients of similar body weights as full-size graft controls (FSG). Implantation surgery was performed as described pre-
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Previously with the hepatic artery and bile duct reconstructed with intraluminal stents [36]. The ratios of graft weight/standard liver weight were not significantly different between QSG with and without IP (P>0.1 by the Student’s t-test). All animals were given humane care in compliance with institutional guidelines using protocols approved by the Institutional Animal Care and Use Committee.

Pulmonary histology

Under pentobarbital (50 mg/kg, ip) anesthesia, rat lungs were harvested at 38 h after implantation and processed for histology [38, 39]. With hematoxylin and eosin (H&E)-stained sections, lung microscopic images were acquired [20]. The thicknesses of alveolar septal walls were quantified by computerized image analysis using IPLab 3.7v software (BD Biosciences, Rockville, MD), as described previously [20], and relative alveolar septal thickness was expressed as a ratio of the average thicknesses of different transplantation groups to the sham-operation group.

Immunohistochemical staining

ED1 (CD68), a marker of monocytes and macrophages, myeloperoxidase (MPO), an indicator of neutrophil infiltration, and intercellular adhesion molecule-1 (ICAM-1) were assessed in deparaffinized pulmonary sections to assess inflammation in lung tissues [20]. Apoptosis was measured by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) using an in situ cell death detection kit (Table 1). TUNEL-positive cells were quantified as described previously [20].

Immunoblotting

Liver and lung tissues were collected at 38 h after transplantation and processed for immunoblotting [20]. Aliquots of liver and lung supernatants (40 μg of protein) were separated on NuPAGE 4-12% Bis-Tris gels, transferred onto nitrocellulose membranes and immunoblotted with primary antibodies specific for ED-1 (CD68), CD4, HSP27, -32, -60, -72, -90, high mobility group box-1 (HMBG-1), MPO, nuclear factor-kB (NF-kB) p65, phospho-NF-kB p65, tumor necrosis factor-α (TNFα), and toll-like receptor-4 (TLR4) at 1:1,000 and actin at 1:3,000 overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies were applied, and detection was accomplished with chemiluminescence.
Detection of tumor necrosis factor-a mRNA by quantitative real-time PCR

Single stranded cDNAs were synthesized from RNA (2 mg) extracted from liver tissue using a Bio-Rad iScript cDNA Synthesis kit, and quantitative real-time PCR (qPCR) was conducted using a CFX96 Real Time-PCR Detection System (Bio-Rad, Hercules, CA) and primer sequences previously described [20].

Serum high mobility group box-1 detection

Under pentobarbital (50 mg/kg, ip) anesthesia, blood samples were collected from the vena cava at 38 h after implantation. Serum HMGB1 was measured using an analytical kit (Table 1) according to the manufacturer’s instructions.

Statistical analysis

Groups were compared using Student’s t-test or ANOVA plus a Student-Newman-Keuls post-hoc test as appropriate. Data shown are means ± SEM. Group sizes were 3-4 livers in each group for all parameters, as indicated in figure legends. Differences were considered significant at P<0.05.

Results

Ischemic preconditioning blunts pro-inflammatory cytokine formation and HMGB1 release after small-for-size liver transplantation

We previously reported that pro-inflammatory cytokine formation increased after small-for-size liver transplantation [20]. Therefore, we investigated whether IP attenuated pro-inflammatory reactions in small-for-size grafts. Full-size grafts (FSG) and quarter-size grafts (QSG) with and without IP were transplanted. TLR4, which mediates pro-inflammatory endotoxin reactions and leads to toxic cytokine formation, was expressed at low levels in livers of sham-operated rats (Figure 1A). Hepatic TLR4 expression did not increase in FSG 38 h after liver transplantation but increased ~15-fold in QSG (Figure 1A, 1B). After IP, TLR4 expression increased only ~9-fold in QSG. Hepatic TNFα protein and mRNA expression also did not increase in FSG but increased markedly in QSG. IP decreased TNFα protein and mRNA expression in QSG by 41% and 43%, respectively (Figure 1C, 1D).

HMGB1 is released during cell stress and injury, acting as a DAMP that promotes inflammatory processes [40, 41]. Previously we reported that QSG transplantation causes mitochondrial dysfunction, increased necrosis and apoptosis,
Ischemic preconditioning decreases NF-κB activation and adhesion molecule expression in recipient lungs after small-for-size liver transplantation

DAMP and pro-inflammatory cytokines released from failing liver grafts has been reported to promote inflammatory reactions in remote organs [43, 44]. Accordingly, we explored activation of NF-κB, which mediates many inflammatory responses of TNFα and HMGB1 [45, 46], in recipient lungs after QSG transplantation. We observed that pulmory NF-κB p65 expression was similar in all treatment groups (data not shown). However, phospho-NF-κB p65, which was barely detectable in lungs after sham-operation and FSG transplantation, increased ~19-fold after QSG transplantation (Figure 2A, 2B). This effect was blunted by IP (Figure 2A, 2B). Similar changes were observed with ICAM-1 which mediates leukocyte adhesion. ICAM-1 expression was primarily in vascular, alveolar and bronchial epithelial cells (Figure 2D and not shown). After IP, pulmonary ICAM-1 decreased after QSG transplantation in comparison to QSG without IP (Figure 2C, 2D).

Ischemic preconditioning decreases heat-shock proteins in recipient lungs after small-for-size liver transplantation

Previous studies show that IP induces HSPs in remote organs and tissues [25, 28, 47, 48]. In rat lungs after QSG transplantation, HSP32, 60 and 72 increased compared to sham operation (Figure 3A, 3C-E). IP before QSG transplantation restored pulmonary HSP 32, 60 and 72.
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Almost to control levels (Figure 3). HSP27 decreased to similar levels in rat lungs after transplantation of QSG with and without IP (Figure 3A, 3B). Pulmonary HSP90 was not altered under any conditions (Figure 3A, 3F).

Ischemic preconditioning decreases alveolar wall thickness and pulmonary inflammation after small-for-size liver transplantation

In FSG recipients, alveolar septa were not thickened compared to sham-operated rats (Figure 4D). In lungs of QSG recipients, alveolar septa thickened 3.7-fold and cellularity in alveolar septa increased markedly (Figure 4B, 4D). Leukocytes, including mononuclear and polymorphonuclear cells, increased overtly in the lungs of QSG recipients. Leukocytes were present in blood vessel lumens and infiltrated into the peribronchial, perivascular and intra-alveolar spaces (Figure 4B and not shown). With IP, alveolar wall thickness only increased to 1.6-fold after QSG transplantation, and pulmonary leukocyte sequestration was substantially less than after QSG transplantation without IP (Figure 4C, 4D).

Adaptive immunity has also been reported to affect the progression of ischemia/reperfusion injury [40, 49], and the inflammatory cytokine TNFα promotes migration of CD4+ T lymphocytes after ischemia/reperfusion [50, 51]. Pulmonary CD4 (a T-cell marker) did not increase after FSG transplantation but increased 6.9-fold after QSG transplantation compared to sham operation (Figure 5D, 5E). By comparison after IP, pulmonary CD4 increased only 4.7-fold following QSG transplantation (Figure 5D, 5E).

No histologically overt necrosis was observed in recipient lungs after QSG transplantation (Figure 4B), and cleaved caspase-8 and caspase-3 were barely detectable in lung tissues of sham-operated rats and recipients of FSG. However after QSG transplantation, pulmonary cleaved caspase-8 and -3 increased substantially in QSG recipients, which IP blunted (Figure 6A-C).

Apoptosis in lung tissue was also detected by TUNEL. TUNEL-positive cells with red nuclear staining were rare (0.3/hpf) in lungs after both
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Discussion

Protection of small-for-size liver grafts by ischemic preconditioning prevents acute pulmonary injury after transplantation

Small-for-size grafts have increased risks of graft failure and postsurgical complications, but exactly how this occurs is uncertain [12, 15]. Pulmonary complications frequently occur after liver transplantation and substantially increase mortality, so prevention/treatment of pulmonary complications is crucial for increasing post-transplantation survival [3, 5, 8]. Our previous data showed that ALI increased markedly in recipients of small-for-size grafts [20]. Moreover, IP of liver grafts prior to harvesting decreased liver injury, improved liver regeneration, and prevented graft dysfunction after small-for-size liver transplantation [35]. Therefore, we explored whether IP also attenuates ALI after small-for-size liver transplantation. In QSG rat recipients, ALI increased markedly (Figures 4-6), a finding that was consistent with our previous observations [20]. Interestingly, hepatic IP in rat liver donors significantly decreased lung injury in recipients after QSG transplantation (Figures 4-6), suggesting the existence of cross-talk between small-for-size liver grafts and recipient lungs.

Ischemic preconditioning did not increase heat-shock proteins in the lung

Many studies reveal that an increase of HSP expression by IP is associated with protection against injuries from ischemia/reperfusion and other stresses [52-55]. We also observed an increase of HSP90 in QSG after transplantation [35]. Interestingly, remote IP can protect organs from injury and decrease systemic inflammation by increasing HSPs [25-28]. For example, hind limb IP protects the myocardium from infarction after ischemia/reperfusion by increasing H0-1 (HSP32) and HSP27 expression in the heart [28, 47] and protects the spinal cord from ischemia/reperfusion injury by increasing HSP70 [48]. Furthermore, IP of one kidney protects the other kidney from subsequent ischemia/reperfusion injury by increasing HSP25 [55]. Similarly, ischemia to one-half of the liver...
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Ischemic preconditioning decreases toxic cytokine and damage-associated molecular pattern molecule release from small-for-size liver grafts

An alternative possibility is that IP decreases the release of toxic mediators from small-for-size liver grafts. Warm hepatic ischemia/reperfusion causes inflammatory responses in remote organs, including the lung [56], and production of pro-inflammatory cytokines TNFα and IL-1β increases in liver grafts exposed to prolonged cold storage, resulting in ALI after liver transplantation [9]. Due to higher portal blood flow, small-for-size liver grafts are exposed to more endotoxin from the gut, which could stimulate Kupffer cells to produce toxic cytokines. Moreover, free radicals, which also stimulate toxic cytokine production, increase substantially in small-for-size grafts [36]. Indeed, we observed that TNFα expression increased in small-for-size liver grafts (Figure 1), consistent with our previous observations [20]. TNFα binds to its receptors to cause pro-inflammatory responses (e.g., NF-κB activation, expression of adhesion molecules, and infiltration of leukocytes) and increase death receptor-mediated caspase activation and apoptosis [46]. IP protects against oxidative stress in QSG [20] which might decrease Kupffer cell activation and toxic cytokine formation. In this study we observed that IP inhibited expression of TLR4, an endotoxin receptor, and decreased TNFα expression in QSG (Figure 1).

Moreover, IP decreased HMGB1 release from QSG (Figure 1). HMGB1 normally resides in nuclei [57, 58], but with stress, HMGB1 translocates to the cytosol to be released. HMGB1 can be released actively by immune cells (e.g., macrophages) or passively by injured and dying cells. HMGB1 is released under diverse conditions, such as shock, endotoxin-induced lethality, ischemia/reperfusion injury, trauma, sterile inflammation, infection, arthritis, and autoimmune disease [57-60]. HMGB1 is a DAMP molecule that has emerged as one of the most important pro-inflammatory mediators as well as an extracellular signaling factor with roles in pathogenesis to many organs/systems [44, 61-66]. HMGB1 binds to membrane receptors.
(TLR4 and advanced glycation end-products receptors), thereby activating NF-κB and promoting inflammatory responses. Administration of recombinant HMGB1 in the mouse trachea causes pulmonary inflammation [67], and serum HMGB1 increases after hepatic ischemia/reperfusion [68, 69], contributing to hepatic warm ischemia/reperfusion-induced acute lung inflammation and injury by binding to pulmonary TLR4 [43]. In patients with acute liver failure, hepatic HMGB1 decreases, whereas plasma HMGB1 increases, indicating HMGB1 release [65]. However, other studies show that although HMGB1 is released from liver during ischemia/reperfusion injury, hepatic HMGB1 increases, possibly due to increased expression [70]. A case report indicates that HMGB1 increases in sera of liver transplantation patients with small-for-size syndrome [71]. In the present work, HMGB1 decreased in QSG but increased in the serum after transplantation, a finding consistent with the conclusion that failing small-for-size grafts were releasing HMGB1. Importantly, IP prevented HMGB1 release from QSG (Figure 1).

Conclusions

Collectively, our data indicate that IP of donor livers decreases ALI after small-for-size liver transplantation, possibly by inhibiting pro-inflammatory cytokine formation and release of DAMP from injured and/or failing grafts. Thus, anti-inflammatory treatment and neutralization of DAMP may be effective for prevention of lung injury, thereby improving outcomes of small-for-size liver transplantation.

Acknowledgements

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Disclosure of conflict of interest

None.

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