Abstract

Objectives: Hepatic ischemia and reperfusion during liver transplant surgery result in hepatocellular damage. Toll-like receptors, especially TLR4, have a fundamental basic role in the inflammatory phase of ischemia-reperfusion injuries. The effect of the TRIF-dependent signaling pathway downstream of TLR4 in hepatic ischemia-reperfusion injury has been well established. However, the role of TLR4-MyD88-dependent signal transduction in hepatic ischemia-reperfusion injury has not yet been clarified. The interferon regulatory factor 5 was introduced as the main regulator of the TLR4-MyD88 signaling pathway for activating proinflammatory cytokines. The present study was carried out to investigate the functional impact of the TLR4/IRF5 signaling axis in hepatic ischemia-reperfusion injury.

Materials and Methods: mRNA expression levels of TLR4, IRF5, tumor necrosis factor α, interleukin 1β, and interleukin 6 were measured using real-time polymerase chain reaction after short (3 h) and long (168 h) reperfusion periods in a hepatic mouse model of ischemia-reperfusion injury in the presence and absence of N-acetylcysteine. Liver damage was evaluated by plasma levels of alanine aminotransferase and histopathology.

Results: Our results show that mRNA levels of TLR4/IRF5 and its downstream cytokines were significantly elevated 3 hours after reperfusion and had drastically fallen to baseline levels 168 hours after reperfusion. Plasma levels of alanine aminotransferase showed the same pattern. Histopathologic study of the samples revealed significant hepatic cell infiltration and necrosis 168 hours after reperfusion. Pretreatment with N-acetylcysteine significantly decreased the mRNA levels of TLR4/IRF5 and its downstream cytokines 3 hours after reperfusion and subsequently improved the previously mentioned hepatic damages 168 hours after reperfusion.

Conclusions: This study suggests a possible role for the TLR4/IRF5 signaling pathway in hepatic ischemia-reperfusion injury. Furthermore, it reveals that N-acetylcysteine may suppress this inflammatory axis and consequently improve hepatic injuries.

Key words: Liver damage, N-acetylcysteine, Reactive oxygen species, Signaling pathway

Introduction

Ischemic shock induced by Pringle maneuver during liver resection followed by subsequent reperfusion after liver transplant leads to warm ischemia-reperfusion injury (IRI). This phenomenon results in complex hepatocellular damage, early graft dysfunction, and eventually increased incidence of acute and chronic rejection.1

Mitochondrial dysfunction due to ischemia-reperfusion shock leads to adenosine triphosphate depletion in some hepatic cells such as Kupffer cells, sinusoidal endothelial cells, and hepatocytes.2 This event forms reactive oxygen species (ROS) in the ischemic liver lobe.2 Reactive oxygen species production damages the endothelial cells and hepatocytes and releases damage-associated molecular patterns, including high-mobility group box 1, heat shock proteins, heparin sulfate, hyaluronan, and so forth.3 Damage-associated molecular patterns can bind to the surface receptors of innate immune cells such as Toll-like receptors (TLRs), especially TLR4. This interaction triggers the inflammatory cascade through which proinflammatory cytokines and chemokines downstream of TLR4 are produced.
Recruitment of neutrophils and T cells and liver necrosis are the final results of this cascade.  

Downstream signaling of TLR4 involves 2 distinct pathways: MyD88-dependent and TRIF-dependent pathways. Several interferon regulatory factors (IRFs) including IRF1, IRF3, IRF5, IRF7, and IRF8 are activated downstream of these pathways. Several reports have investigated the roles of IRF1 and IRF3 in liver IRI. However, to the best of our knowledge, there are no data supporting the involvement of IRF5 during liver IRI. Therefore, this study was carried out to investigate the role of the TLR4/IRF5 signaling pathway in the hepatic IRI model, by evaluating mRNA expression levels of TLR4/IRF5 axis and its downstream genes, including tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and interleukin 1β (IL-1β). We also explored the effect of N-acetylcysteine (NAC), a potent antioxidant drug, on this signaling pathway and its mechanism of action in preventing ischemia-reperfusion-induced liver injury.

Materials and Methods

Liver ischemia

Male Balb/c mice (8- to 10-week-old; 26-30 g) were purchased from Shiraz University of Medical Sciences (Shiraz, Iran). Mice were housed in plastic cages with free access to food and water. The mice were randomly placed in groups subjected to sham operation, control conditions, or IRI, which consisted of 60-minute ischemia followed by 3-hour and 168-hour reperfusion (10 mice/group). N-acetylcysteine (C5H9-NO3S, Sigma-Aldrich, St. Louis, MO, USA) was injected intravenously (150 mg/kg) through the tail vein from 1 hour before ischemia until 3 hours and 168 hours after reperfusion (6 mice/group). As previously described, animals were anesthetized using 100 mg/kg ketamine and 10 mg/kg xylazine intraperitoneally for partial (70%) hepatic ischemia. A midline laparotomy was performed, and portal triads from left and median lobes were occluded using an atraumatic microvascular clamp. After 60 minutes, the ischemia clamp was removed to allow hepatic reperfusion. Abdomens were then closed using 5-0 silk sutures. Sham operation mice underwent the same protocol without portal triad occlusion. Control group mice were anesthetized and humanely killed without any operation. Blood samples were collected from the left ventricle. Median and left liver lobes were frozen at -80°C for further analyses. All animal protocols were approved by the Ethics Committee of Shiraz University.

Real-time polymerase chain reaction

Total RNA was isolated from frozen samples of liver using the RNX-Plus kit (CinnaGen, Tehran, Iran) according to the manufacturer’s instruction. RNA samples were reverse transcribed into cDNA using the PrimeScript RT reagent Kit (Takara, Shiga, Japan). Relative mRNA expression of each gene was evaluated with the use of the Rotor-gene 6000 real-time polymerase chain reaction system (Corbett Life Science, Sydney, Australia) using the SYBR Green Premix Ex Taq II kit (Takara). Primer sequences were designed using AlleleID version 7.5 software (PREMIER Biosoft, Palo Alto, CA, USA) (Table 1). All primer pairs were specific for mRNA and were tested for no amplification of genomic DNA. Polymerase chain reactions were performed in 10 μL of final volume (containing 50 ng cDNA) and set at 95°C for 30 seconds, followed by 45 cycles at 95°C for 20 seconds, 60°C for 45 seconds for TLR4, TNF-α, IL-1β, IL-6, and eukaryotic elongation factor 1 (EF-1) and also 95°C for 30 seconds, followed by 45 cycles of 95°C for 20 seconds, 62°C for 30 seconds, and 72°C for 30 seconds for IRF5 and EF-1. Expression levels of each gene were first normalized with EF-1 (as

Table 1. Primer Sequences and Amplicons

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Forward (5' → 3')</th>
<th>Reverse (5' → 3')</th>
<th>Amplicon size, base pair</th>
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<td>CAGAGGACCAACCACCTGAT</td>
<td>GTACGGCGAACCTGATGTC</td>
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<tr>
<td>TNF-α</td>
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<td>CAGGCTCTTTCGACCTGA</td>
<td>ACCTGGTGTGCTGACAG</td>
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<tr>
<td>IL-1β</td>
<td>NM_008361</td>
<td>TGACATGCTAGGAAATGGCT</td>
<td>GCTGCTGAGAATTTGAA</td>
<td>122</td>
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<tr>
<td>IL-6</td>
<td>NM_0031164</td>
<td>GCAGATGCGACATTCTGATG</td>
<td>TGCTTCAATGATGACAGTTG</td>
<td>180</td>
</tr>
<tr>
<td>EF-1</td>
<td>NM_010106</td>
<td>AGTCGCCCGCGCCTTTCT</td>
<td>CGATTAGACGACGAGTGT</td>
<td>124</td>
</tr>
</tbody>
</table>

Abbreviations: EF-1, eukaryotic elongation factor 1; IL, interleukin; IRF5, interferon regulatory factor 5; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor alpha
internal control) and compared with the sham group using the 2-ΔΔCt method.

Liver damage assessment
To evaluate the hepatic function and cellular injury after liver ischemia, plasma levels of alanine aminotransferase were measured using an autoanalyzer (Mindray BS-380, Chema, Monsano, Italy). Formalin-fixed samples from left lobes of livers were embedded in paraffin. Hepatic sections (5 μm) were stained with hematoxylin and eosin. Nine sections from each mouse were scored by an expert pathologist from 0 to 4 for their sinusoidal congestion, hepatocyte vacuolation, polymorphonuclear cell infiltration, and parenchymal necrosis, as described by Suzuki and associates.10 The observer was blinded to treatment groups.

Statistical analyses
Data are presented as means ± standard error of the mean. Multiple comparisons between groups were performed using analysis of variance with Bonferroni post hoc test. The independent sample t test was applied to determine differences between pretreatment groups with and without NAC. Suzuki scores were analyzed with the Mann-Whitney U test.

Results
mRNA expression levels of TLR4/IRF5 signaling axis and their downstream proinflammatory cytokine genes in hepatic ischemia-reperfusion injury
Evaluating mRNA levels of TLR4 and IRF5 showed significant overexpression of TLR4 (P < .001) and IRF5 (P = .019) 3 hours after reperfusion compared with the sham-operation group. At 168 hours after reperfusion, mRNA levels of both genes returned to the same baseline levels as in the sham group (Figure 1A and 1B).

We measured mRNA levels of the 3 major downstream cytokines of IRF5 to follow the activation of TLR4/IRF5 signaling pathway in hepatic IRI. Our results showed that mRNA expression levels of TNF-α (P = .001), IL-6 (P < .001), and IL-1β (P < .001) were increased in the 3-hour reperfusion group compared with the sham group. Furthermore, mRNA levels returned to sham levels after 168 hours of reperfusion (Figure 1C-1E). There were no significant differences between control and sham-operation group for mRNA levels of all examined genes (Figure 1).

N-acetylcysteine, a potent ROS scavenger, was injected 1 hour before ischemia to investigate the role
of ROS release on activation of TLR4/IRF5 signaling pathway in our IRI model. Our results indicated that pretreatment with 150 mg/kg of NAC could significantly downregulate the mRNA expression of TLR4 \((P = .004)\), IRF5 \((P = .008)\), and its downstream cytokines TNF-α \((P = .014)\), IL-6 \((P = .041)\), and IL-1β \((P < .001)\) 3 hours after reperfusion (Figure 1).

Plasma levels of alanine aminotransferase and liver histology after hepatic ischemia-reperfusion injury

Plasma levels of alanine aminotransferase were increased 3 hours after reperfusion compared with the sham group \((P < .001)\) and subsequently returned to the sham level 168 hours after reperfusion, indicating the acute hepatocyte response to IRI (Figure 2A).

As shown in Figure 2B, the pathologic study of hepatic tissues based on the Suzuki scores revealed significant increases in sinusoidal congestion \((P = .008)\), hepatocyte necrosis \((P < .002)\), and polymorphonuclear cell infiltration \((P = .003)\) 168 hours after reperfusion compared with the sham group. However, no significant hepatocellular damage was observed 3 hours after reperfusion time compared with the sham group. Furthermore, our results indicated that the use of NAC obviously improved sinusoidal congestion \((P = .025)\), hepatocellular necrosis \((P = .026)\), and polymorphonuclear cell infiltration \((P = .024)\) 168 hours after reperfusion (Figure 2C).

Discussion

It has been shown that IRF5 is a major regulator of proinflammatory cytokines in the MyD88-dependent signaling pathway of TLRs and has a critical role in promotion of inflammatory macrophage polarization.\(^6,\)\(^1\)\(^1\)\(^-\)\(^1\)\(^3\) Therefore, a potential role in initial inflammatory phase of hepatic IRI can be suggested for IRF5. On the basis of previous findings, we aimed to evaluate the expression profile of TLR4/IRF5 axis in hepatic IRI.

Our results demonstrated that TLR4, IRF5, and their downstream cytokines (TNF-α, IL-6, and IL-1β) were transcriptionally activated at the initial inflammatory phase of hepatic IRI (3-h reperfusion) and subsequently returned to baseline levels 168 hours after reperfusion. These results correspond well with a previous study, which demonstrated that IRF5 is overexpressed in the inflammatory phase of coronary ligation in mice and falls drastically to baseline levels in the necrotic phase.\(^1\)\(^4\) Up-regulation of the TLR4/IRF5 signaling pathway confirmed the previous findings, indicating the activation of Kupffer cells during early reperfusion time of hepatic IRI. Kupffer cell activation induces proinflammatory cytokine production and leads to widespread effects in the initiation and propagation of IRI.\(^1\)\(^2\) In addition, their down-regulation may be a result of activated negative regulatory responses after the initial burst of TLR4-mediated signaling.\(^1\)\(^5\)

Furthermore, we showed that NAC could transcriptionally repress the up-regulation of all examined genes in this pathway (Figure 1). As shown by our histologic findings, NAC pretreatment led to an approximately 52% reduction of hepatic IRI.
The antioxidant activity of NAC was accomplished through preventing the formation of ROS and also by scavenging ROS once formed. Our findings confirmed the previous studies indicating that NAC treatment impairs the chemotaxis and ROS production in human phagocytic cells and early TLR4 activation, which is a ROS-dependent event. A previous study by Wang and associates showed that NAC can prevent hepatic IRI-induced autophagy and apoptosis by influencing the Janus kinase signaling pathway. The effect of NAC on suppressing the TLR4/IRF5 axis and its downstream proinflammatory cytokines suggests another possible mechanism of action for preventing hepatic IRI. Findings from our present study show that activation of the TLR4/IRF5 signaling axis may have an essential role in the early inflammatory phase of hepatic IRI. Suppression of this inflammatory signaling axis by NAC suggests another possible mechanism for improvement of hepatic IRI. Altogether, it seems that IRF5 can be a good target for reducing liver damage caused by hepatic ischemia-reperfusion during liver transplant surgery.

References