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Nrf2 activation protects the liver from ischemia/ reperfusion injury in mice

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Abstract

Objective—To investigate the role of Nrf2 in the pathogenesis of hepatic ischemia-reperfusion (I/R) injury.

Summary Background Data—Hepatic I/R injury is a serious complication that leads to liver failure after liver surgery. NF-E2-related factor 2 (Nrf2) is a transcription factor that plays a critical role in protecting cells against oxidative stress. Therefore, it is suggested that Nrf2 activation protects the liver from I/R injury.

Methods—Wild-type (WT) and Nrf2-deficient mice were treated with 15-deoxy-^{12, 14}-prostaglandin J₂ (15d-PGJ₂), or a vehicle. Subsequently, these mice were subjected to 60 min hepatic 70% ischemia followed by reperfusion. Liver and blood samples were collected to evaluate liver injury and mRNA expressions.

Results—After hepatic I/R, Nrf2-deficient livers exhibited enhanced tissue damage, impaired GSTm1, NQO1, and GCLC inductions, disturbed redox state, and aggravated TNF- α mRNA expression in comparison to WT livers. 15d-PGJ₂ treatment protected the livers of WT mice from I/R injury via increased expressions of GSTm1, NQO1 and GCLC, maintained redox status, and decreased TNF- α induction. These effects induced by 15d-PGJ₂ were not seen in the livers of Nrf2^{-/-} mice and were not annulled by PPAR γ antagonist in Nrf2^{+/+} mice, suggesting that the protective effect of 15d-PGJ₂ is mediated by Nrf2-dependent antioxidant response.

Conclusions—Nrf2 plays a critical role in the mechanism of hepatic I/R injury and would be a new therapeutic target for preventing hepatic I/R injury during liver surgery.

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Introduction

Interruption of hepatic blood inflow to decrease blood loss during liver surgery such as hepatic resection and transplantation causes hepatic ischemia and subsequent reperfusion that result in massive hepatocyte injuries. Ischemia-reperfusion (I/R) liver injury is a severe, unfavorable postsurgical complication associated with high morbidity and mortality. A number of studies have demonstrated that generation of reactive oxygen species (ROS) is associated with hepatic I/R injury.^{1–4} During the early phase of I/R, ROS causes hepatocyte damage through lipid peroxidation, protein oxidation, mitochondrial dysfunction, and DNA damage.^{2, 5} Subsequently, Kupffer cells and accumulated neutrophils are activated in response to hepatocyte death and cause liver inflammation.³ Thus, regulation of ROS is suggested as a new therapeutic strategy for hepatic I/R injury.

Nrf2 (NF-E2-related factor 2) is a transcription factor associated with various intracellular signaling that protects organs against oxidative stress.^{6–11} In physiological conditions, Nrf2 is retained in cytoplasm by binding to its inhibitor, Keap1. Various endogenous or exogenous stimuli dissociate Nrf2 from Keap1 that leads to the nuclear translocation of Nrf2, resulting in transcriptional activation of antioxidant responsive element (ARE)-regulated genes, such as glutathione-S-transferases (GSTs), NADPH, quinine oxidoreductase 1 (NQO1), and glutamate cysteine ligase (GCL).¹² A number of studies have shown that depletion of Nrf2 increases susceptibility to toxin-induced liver injury,^{13–16} all of which provide strong evidence for Nrf2 as a hepatoprotective factor for liver injury. However, the involvement of Nrf2 in hepatic I/R injury has not been investigated to date.

Here, we demonstrate that Nrf2 plays a crucial role in the protection of hepatic I/R injury. We also found that treatment with 15-deoxy-^{12,14}-prostaglandin J2 (15d-PGJ2)—a derivative of omega-6 polyunsaturated fatty acids that is produced from the non-enzymatic dehydration of PGD2¹⁷—protected livers from I/R injury via activation of Nrf2. Our results provide insight into the amplification of Nrf2 activation as a powerful interventional strategy to protect livers from I/R insults during and after surgical procedures.

Materials and Methods

Model of Hepatic Ischemia/ Reperfusion Injury

Male 9 to 11-week-old wild-type (WT) male mice (C57BL/6 mice; Japan SLC, Tokyo, Japan) and Nrf2 knockout male mice on C57BL/6 background were used in this study. Nrf2 knockout mice/C57BL6J (RBRC01390) were provided by RIKEN BRC, which is participating in the national Bio-Resource Project of the MEXT, Japan. The protocol for animal experiments in this study has been approved by the Animal Research Committee in Akita University (approval number: a-1-2213). All subsequent animal experiments adhered to the “Regulation for Animal Experimentation,” of the Akita University. Mice were anesthetized with pentobarbital sodium. After midline laparotomy (2cm), partial hepatic ischemia was induced by clamping the vessels to the left and median lobes of the liver using an atraumatic clip to hinder blood supply to the liver. After a 60-minute ischemia, the clip was removed to accomplish reperfusion. The abdomen was closed in layers, and the animals were allowed to recover in their cages. Some mice were injected intravenously with vehicle

(10% DMSO) or 0.3mg/kg 15d-PGJ2 (Enzo Chemical Co., St. Louis, MO) 3 hours prior to ischemia. To block PPAR γ activity, a separate group of mice was intraperitoneally injected with 1.0mg/kg of GW9662 (Wako, Osaka, Japan) 30 minutes prior to 15d-PGJ2 administration, as described elsewhere.¹⁸ At several indicated time points, mice were sacrificed to collect the liver and blood samples.

Serum enzyme analyses

Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by FUJI DRI-CHEM SLIDE system (Fujifilm, Tokyo, Japan).

Histopathology

Liver samples were fixed in 4% paraformaldehyde and embedded in paraffin. Liver sections were stained with hematoxylin and eosin.

Isolation and culture of primary hepatocytes

Primary hepatocytes were isolated from WT C57BL/6 mice and cultured as described previously.¹⁹

Immunofluorescence

Primary hepatocytes were stimulated with 6 μ M 15d-PGJ2 or DMSO for 1 hour. For Nrf2 detection, cells were fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. Subsequently, cells were blocked with 5% BSA in PBS and incubated overnight with rabbit polyclonal anti-Nrf2 antibody (1:50 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C. After washing, cells were incubated with Alexa Flour 594-conjugated chicken anti-rabbit IgG (Invitrogen, Carlsbad, CA). DAPI was used for nuclear staining. After washing, cells were mounted in fluorescent mounting medium and examined using the confocal microscope (Zeiss, Jena, Germany).

Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA extraction and real-time RT-PCR were performed as described previously.¹⁹ The primer sequences for RT-PCR were shown in Supplemental table1.

Western blot analysis

Whole liver extracts were prepared and subjected to Western blot analysis as previously described,¹⁹ using antibodies specific for NQO1, GCL catalytic subunit (GCLc) (Abcam; Cambridge, UK), and β -actin (Sigma, St Lois, MO).

TdT-Mediated dUTP Nick-end Labeling (TUNEL) Assay

Terminal deoxynucleotidyl transferase-mediated dUTP-nick-end labeling (TUNEL) was performed using an “*in situ* cell detection kit” (Roche) according to the manufacture’s instructions. The percentage of TUNEL-positive hepatocytes represents the number of TUNEL-positive hepatocytes/ total number of hepatocytes in five randomly chosen 400 $\times$ fields on each slide.

Glutathione analysis

Glutathione levels of the liver tissue were measured by using Glutathione Assay Kit (Cayman Chemical company, Ann Arbor, MI) according to the manufacture's instructions.

MDA analysis

Malondialdehyde (MDA) concentrations of the liver tissue were measured by the NWLSS Malondialdehyde assay kit (Northwest Life Sciences Specialities, Vancouver, Canada) according to the manufacture's instructions.

Statistics

Data are expressed as means $\pm$ SEM. Student's 2-tailed *t* test was used to evaluate the differences between the vehicle- and 15d-PGJ2-treated mice within a single genotype as well as between genotypes. In addition, the difference between before and after I/R of the vehicle-treated mice was also evaluated. *P* < 0.05 was considered statistically significant.

Results

Hepatic I/R injury was exacerbated in Nrf2^{-/-} mice and prevented by the treatment with 15d-PGJ2 in WT mice

To investigate the role of Nrf2 on hepatic I/R injury, we used Nrf2^{-/-} mice and their Nrf2^{+/+} littermates as WT control. Serum AST and ALT levels were significantly elevated in Nrf2^{-/-} mice compared to Nrf2^{+/+} mice after 6 hours of reperfusion (AST: 1758 $\pm$ 376 vs 857 IU/L $\pm$ 84 IU/L, *p* < 0.05; ALT: 2893 $\pm$ 450 IU/L vs 1835 $\pm$ 158 IU/L, *p* < 0.05). Since this result suggested a preventive role of Nrf2 in the progression of hepatic I/R injury, we hypothesized that amplification of Nrf2 would have a therapeutic effect on hepatic I/R injury. To test this hypothesis, Nrf2^{+/+} and Nrf2^{-/-} mice were treated with 15d-PGJ2—an Nrf2 activator—before I/R (Fig. 1A). In Nrf2^{+/+} mice, 15d-PGJ2 pretreatment significantly reduced the elevation of serum AST and ALT levels 6 hours after reperfusion. In contrast, treatment with 15d-PGJ2 had no effect on the elevation of serum transaminase levels in Nrf2^{-/-} mice following hepatic I/R. We further examined whether treatment with 15d-PGJ2 activates Nrf2 in primary hepatocytes using immunofluorescence for Nrf2 (Fig. 1B). DMSO-treated hepatocytes showed diffuse and faint staining, whereas the cells treated with 15d-PGJ2 had a strong staining for Nrf2 in the nucleus, demonstrating the induction of nuclear translocation of Nrf2 by 15d-PGJ2 treatment. These results indicate that pre-administration of 15d-PGJ2 activates Nrf2 and protects livers against I/R injury in an Nrf2-dependent manner.

Oncotic necrosis was enhanced in Nrf2^{-/-} mice

Histology was then assessed to evaluate the degree of cell death in each mouse. H&E staining of WT livers 6 hours after reperfusion exhibited gross necrosis of hepatocytes characterized by cell swelling, karyolysis, loss of basophilic staining, and vacuolization in centrilobular area (Fig. 2A). A number of nuclei and cytoplasm of hepatocytes were positive for TUNEL staining (Fig. 2B). The massive DNA fragmentation in hepatocytes suggests apoptosis and necrosis. Consistent with increased serum levels of liver enzymes in Nrf2^{-/-}

mice after I/R, the area of necrosis and the number of TUNEL-positive cells were greater in Nrf2^{-/-} livers than Nrf2^{+/+} livers (Fig. 2C). In addition, treatment with 15d-PGJ2 markedly reduced the extent of necrosis and TUNEL-positive hepatocytes in Nrf2^{+/+} mice, but not in Nrf2^{-/-} mice. These results indicate that Nrf2 plays a protective role and its preactivation has a therapeutic effect on hepatic I/R injury.

Expressions of antioxidant enzymes were reduced in Nrf2^{-/-} mice

To examine hepatic Nrf2 activation, we measured mRNA levels of Nrf2-dependent antioxidant genes including GST Mu1 (GSTm1), NQO1, and GCLC in the liver. Before ischemia, the expressions of GSTm1, NQO1, and GCLC mRNA were significantly reduced in the livers of Nrf2^{-/-} mice compared with those of Nrf2^{+/+} mice (Fig. 3A, B, and C). The expressions of these mRNA were significantly increased in 15d-PGJ2-treated Nrf2^{+/+} livers, but were not altered in 15d-PGJ2-treated Nrf2^{-/-} livers. These results indicate that 15d-PGJ2-mediated Nrf2 activation induces the expressions of GSTm1, NQO1, and GCLC. Three hours after reperfusion, the expressions of these mRNA were markedly elevated in Nrf2^{+/+} livers; however, no differences in mRNA levels were observed between the vehicle- and 15d-PGJ2-treated Nrf2^{+/+} mice. In Nrf2^{-/-} livers, however, the elevations of these genes after reperfusion were not significant either in vehicle- or in 15d-PGJ2-treated mice. These results indicate that hepatic I/R stimulate Nrf2 activation and the following induction of GSTm1, NQO1, and GCLC mRNA expressions. To confirm whether the levels of these mRNAs correlate with their respective protein expressions, the protein levels of NQO1 and GCLC were investigated as representative Nrf2-target antioxidant enzymes (Fig.3E). Western blot shows consistent kinetics between these two protein expressions and mRNA expressions.

We also measured the mRNA expression of Heme oxygenase 1 (HO-1)—a well-known protective gene for hepatic I/R injury— because HO-1 has also been reported to be one of Nrf2-target genes.^{11, 20, 21} However, the expression pattern of HO-1 mRNA was completely different from those of GSTm1, NQO1, and GCLC mRNA (Fig. 3D). Basal levels were similar in two genotypes, while the expression was upregulated to a greater extent in Nrf2^{-/-} mice after hepatic I/R. In addition, 15d-PGJ2 treatment did not affect the expression of HO-1. These results indicate that HO-1 is induced by Nrf2-independent mechanisms.

Oxidative stress and TNF- α expression were aggravated in Nrf2^{-/-} mice

To investigate the redox state of the livers, we measured GSH and GSSG contents in the liver (Supplemental Table 2). Hepatic GSH levels decreased 3 hours after reperfusion in all groups. Six hours following reperfusion, GSH levels recovered slightly in Nrf2^{+/+} livers, whereas in Nrf2^{-/-} livers, the decreased GSH levels sustained. Six hours after reperfusion, the GSH levels of Nrf2^{+/+} livers treated with 15d-PGJ2 recovered to that of a normal level under healthy conditions. However, no such recovery in GSH levels were observed in 15d-PGJ2 treated Nrf2^{-/-} livers 6 hours following reperfusion. While hepatic GSSG levels were increased in all groups following I/R, Nrf2^{+/+} mice had lower GSSG levels than Nrf2^{-/-} mice after reperfusion, although the difference did not reach statistical significance. In addition, 15d-PGJ2 did not affect hepatic GSSG levels in both genotypes of mice. For better understanding of oxidative stress status in the livers of each mouse, the GSH/GSSG ratio

and hepatic levels of MDA—a biomarker of lipid peroxidation—were evaluated (Fig. 4A and B). Under normal conditions, no significant differences in the hepatic GSH/GSSG ratio and MDA level were observed between the 2 groups. However, decrease in the GSH/GSSG ratio and increase in the MDA level after hepatic I/R were significantly greater in Nrf2^{-/-} mice than in Nrf2^{+/+} mice, suggesting that Nrf2 deficiency leads to the augmentation of oxidative stress induced by I/R. Treatment with 15d-PGJ2 significantly suppressed the decrease in GSH/GSSG ratio and increase in MDA levels observed after reperfusion in Nrf2^{+/+} mice, but not in Nrf2^{-/-} mice. These results suggest that activation of Nrf2 is critical to prevent oxidative stress during hepatic I/R.

Because oxidative stress activates a redox-sensitive transcription factor NF-κB and the activation of NF-κB induces the expression of proinflammatory mediators, we assessed the changes of TNF-α mRNA expression during hepatic I/R. Basal expression levels of TNF-α were similar between the 2 groups. Six hours after reperfusion, TNF-α expression was significantly increased in Nrf2^{-/-} mice, but was suppressed in 15d-PGJ2-pretreated Nrf2^{+/+} mice (Fig. 4C). These results suggest that Nrf2 plays an important role in prevention of excessive inflammatory response after reperfusion.

The expression of PPARγ was reduced in Nrf2^{-/-} mice

Peroxisome proliferator-activated receptors (PPARs) are the members of the nuclear hormone receptor superfamily and classified into three isoforms, α, β, and γ.¹⁷ Of these three isoforms, PPARγ, is known to have an anti-inflammatory effect. Because 15d-PGJ2 is a natural ligand for PPARγ, we measured the hepatic expression of PPARγ mRNA in each group. The levels of PPARγ mRNA were significantly lower in Nrf2^{-/-} mice than in Nrf2^{+/+} mice (Fig. 5A). In addition, 15d-PGJ2 treatment significantly enhanced the mRNA expression of PPARγ in Nrf2^{+/+} mice, but not in Nrf2^{-/-} mice, suggesting that the expression of PPARγ is, at least in part, Nrf2-dependent. To evaluate whether protective effect of 15d-PGJ2 against hepatic I/R was mediated by PPARγ enhancement, 15d-PGJ2-injected Nrf2^{+/+} mice were pretreated with GW9662—a selective PPARγ antagonist—to block PPARγ activity. GW9662 suppressed PPARγ activation: pretreatment with GW9662 inhibited upregulation of PPARγ target genes—CD36, ATP-binding cassette transporter A1 (ABCA1), ABCG1, and liver X receptor α (LXRα)—uniformly in the liver of 15d-PGJ2-treated Nrf2^{+/+} mice (Fig. 5B). In spite of effective blocking of PPARγ, serum AST and ALT levels after hepatic I/R were not different from those without GW9662 pretreatment (AST: 878 ± 259 IU/L with GW9662 vs 906 ± 139 IU/L without GW9662, *p* > 0.05; ALT: 1240 ± 254 IU/L with GW9662 vs 1360 ± 314 IU/L without GW9662, *p* > 0.05, *n* = 5 per group). These results indicate that 15d-PGJ2 indeed enhance the expression and activation of PPARγ in an Nrf2-dependent manner, however the protective function of 15d-PGJ2 against hepatic I/R is not PPARγ-dependent.

Discussion

Numerous studies have revealed strong evidence for critical involvement of ROS in hepatic I/R injury.^{1, 3, 4, 23} Therefore, inhibition of ROS production and scavenging ROS have been expected as therapeutic approaches. Nrf2—a transcription factor mediating the expression

of many endogenous antioxidants—is known to play an important role in the cytoprotection against oxidative stress. Recent studies have shown that Nrf2 also exerts anti-inflammatory effect through regulation of NADPH oxidase and pro-inflammatory signaling.^{7, 24, 25} An accumulating number of reports have revealed that Nrf2-null mice are highly susceptible to hepatic injuries induced by chemicals, methionine-choline-deficient diet, sepsis, and cytokines, indicating that Nrf2 protects the liver through multiple cytoprotective pathways.^{7, 13, 14} From this perspective, it is reasonable to establish therapeutic strategies targeting Nrf2. In this study, we demonstrate that Nrf2 confers the livers with protection against I/R. During reperfusion phase, GSTm1, NQO1, and GCLs—representative Nrf2-target genes which are reported to play protective role as an antioxidant and/or anti-inflammatory molecule in various diseases^{12, 26}—were markedly increased in the livers of Nrf2^{+/+} mice. In contrast, in the livers of Nrf2^{-/-} mice, the induction of these mRNAs and protein were inhibited; instead, accentuation of liver damage, the deterioration of oxidative stress, and excess expression of TNF- α mRNA were seen. Collectively, these results strongly suggest that Nrf2 activation during hepatic I/R functions to eliminate ROS and suppress inflammatory response through induction of these antioxidant genes.

We also demonstrated that 15d-PGJ2 administration reduce oxidative stress and TNF- α mRNA expression that consequent in less hepatic damage upon hepatic I/R in Nrf2^{+/+} mice. This beneficial effect of 15d-PGJ2 was not seen in Nrf2^{-/-} mice, suggesting that the protective effect of 15d-PGJ2 is Nrf2 dependent. Given the ability of Nrf2 to regulate wide array of antioxidant genes, it is conceivable that induction of multiple Nrf2-responsive genes before ischemic insult is sufficient to protect liver damage caused by I/R. In our study, GSTm1, NQO1, and GCLc mRNA expression were significantly upregulated in 15d-PGJ2-treated Nrf2^{+/+} mice prior to ischemia, although the expression of these genes reached to similar extent after I/R. The expressions of NQO1 and GCLc proteins were also increased by 15d-PGJ2 in Nrf2^{+/+} livers before ischemia and were maintained high level after reperfusion. We observed remarkable differences in hepatocyte protection after I/R between the mice treated with and without 15d-PGJ2. Therefore, production of antioxidant proteins driven by upregulation of antioxidant genes before ischemia through Nrf2 preactivation might contribute to the protective mechanism.

It is well known that increased ROS triggers the onset of mitochondrial permeability transition, resulting in the induction of both apoptotic and necrotic cell death.²⁷ Previous studies revealed that oncotic necrosis is predominant mode of cell death after I/R of the liver because ATP depletion blocks apoptotic signaling, which is required for caspase activation.^{27, 28} In agreement with previous reports, damaged hepatocytes were diffusely stained by TUNEL assay, and these hepatocytes did not meet the morphological criteria of apoptosis (cell shrinkage, chromatic condensation and margination, and apoptotic bodies) in our study. In addition, TUNEL positive hepatocytes were located in the confluent area around pericentral regions where oxygen supply is low. These findings are characteristic in the histology for oncotic necrosis of the liver tissue. It has been reported that this type of cell death is also observed in acetaminophen overdose-mediated liver injury.²⁹ The formation of *N*-acetyl-*p*-benzoquinone imine—a cytotoxic reactive metabolite, which leads to GSH consumption, mitochondrial damage, ATP depletion, and ROS formation—has been

postulated to be one of the mechanisms of oncotic necrosis. Because the activation of Nrf2 is also known to protect against acetaminophen-induced hepatotoxicity,^{13, 16, 30} it is suggested that Nrf2 may have an ability to confer the liver with protection in various pathological conditions that cause oncotic necrosis through oxidative stress.

GSH is the most abundant antioxidant in hepatocytes and plays a central role in maintaining redox homeostasis.^{31, 32} Because GSH can detoxify ROS such as hydrogen peroxide, hypochlorous acid, and peroxynitrite, GSH is expected to protect the organs from oxidative stress.^{33, 34} N-acetylcysteine (NAC)—a thiol-containing synthetic compound that increases intracellular GSH level—is mainly used for treating acetaminophen poisoning.^{35, 36} NAC has also been reported to replenish hepatic GSH store, thereby reducing hepatic I/R injury in many experimental studies. However, there is no clinical evidence stating that the use of NAC is effective as a protective agent to attenuate liver injury in patients undergoing liver surgery. One explanation of this discrepancy is that glutathione has a very short half-life in human livers.³⁷ Other antioxidative strategies against hepatic I/R, the use of exogenous antioxidant enzymes such as catalase or superoxide dismutase has also been investigated.⁴ Since these enzymes cannot maintain their bioavailability when administered intravenously, the protective effect has been proved using gene delivery system, which is difficult to apply clinically with present technologies. In this study, we showed that the expression levels of GCLC—GSH-synthesizing enzyme—was higher in Nrf2^{+/+} mice than in Nrf2^{-/-} mice. GCLC level was further increased by the pretreatment with 15d-PGJ2 in Nrf2^{+/+} livers. Consistent with the level of GCLC expression after reperfusion, GSH content was sustained low in Nrf2^{-/-} mice and recovered well in 15d-PGJ2-pretreated Nrf2^{+/+} mice. This fact suggests that Nrf2-dependent induction of GCLC confers effective regulation of GSH.

As mentioned above, we showed that 15d-PGJ2 suppressed hepatic I/R injury in an Nrf2-dependent manner. The function of 15d-PGJ2 was initially identified as a natural endogenous ligand for PPAR γ .¹⁷ Among various functions of PPAR γ , the protective properties of PPAR γ against hepatic I/R was reported by Kuboki et al.³⁸ They demonstrated that administration of rosiglitazone (synthetic PPAR γ agonist) or C-peptide (PPAR γ activator) maintained PPAR γ activity during I/R and reduced liver damage, suggesting the protective role of PPAR γ activation against I/R injury. They further showed that in hepatic I/R injury, the hepatic content of endogenous 15d-PGJ2 was reduced in association with the reduced activation of PPAR γ . Our data show that the expression of PPAR γ mRNA was significantly higher in the liver of Nrf2^{+/+} mice than Nrf2^{-/-} mice and was increased by 15d-PGJ2 treatment only in the liver of Nrf2^{+/+} mice. A comparable results were also reported previously by Cho et al.³⁹ in the lung, corroborating our data showing Nrf2 dependency of PPAR γ expression. In addition, we demonstrated that 15d-PGJ2 increased hepatic mRNA expressions of CD36, ABCA1, ABCG1, and LXR α —representative PPAR γ target genes—, indicating that 15d-PGJ2 increased transcriptional activity of PPAR γ in the liver of Nrf2^{+/+} mice before ischemia. Therefore, it was speculated that the hepatoprotective function of exogenous 15d-PGJ2 observed in the current study was also mediated by PPAR γ via Nrf2 activation. While pretreatment with a PPAR γ antagonist, GW9662, reduced the 15d-PGJ2-dependent upregulation of PPAR γ target genes, it did not annul the protective effect of exogenous 15d-PGJ2 against hepatic I/R in Nrf2^{+/+} mice. This finding implies that PPAR γ is dispensable for 15d-PGJ2-mediated liver protection in our model, but protective effect of

15d-PGJ2 on I/R injury is undoubtedly Nrf2-dependent. As a reason for this, the following mechanism is conceivable. Because protective function of PPAR γ is considered to be mediated by transrepression of the inflammatory transcription factors,^{39,40} continuous activation of PPAR γ during I/R might be necessary to suppress inflammatory response. However, 15d-PGJ2 has a very short half-life and hepatic 15d-PGJ2 level is rapidly reduced during I/R.^{38, 41} Therefore, there is a strong likelihood that exogenous 15d-PGJ2 enhancing PPAR γ activity is rapidly decreased during hepatic I/R, failing to upkeep PPAR γ activation and accordingly, its subsequent transrepression of inflammatory response. Overall, our findings indicate that the mechanism of the liver protection with exogenous 15d-PGJ2 is not mediated by PPAR γ activation but other pathway under Nrf2 activation, in which we demonstrated the importance of antioxidant preinduction.

HO-1 does not seem important in Nrf2 preactivation protocol for liver protection. It has been suggested that HO-1 is an Nrf2-dependent gene in certain cells,^{20, 21} but some studies showed a significant induction of HO-1 in the liver of Nrf2^{-/-} mice.⁴²⁻⁴⁴ In the present study, the expression of HO-1 mRNA did not respond to 15d-PGJ2 treatment even in Nrf2^{+/+} mice before ischemia. After hepatic I/R, HO-1 mRNA upregulation was more marked in Nrf2^{-/-} mice than in Nrf2^{+/+} mice, while it did not contribute to hepatocyte protection. This finding suggests that the upregulation of HO-1 mRNA in this study are merely a sequel to I/R damage. For HO-1, in addition to ARE, the regulatory region of HO-1 contains various binding sites for transcription factors such as NF- κ B and AP-1, which are activated during hepatic I/R.^{4, 45, 46} The complex regulatory mechanism of HO-1 expression might influence the behavior of HO-1, which is distinct from that of GSTm1, NQO1, or GCLs in our model.

Our current study demonstrates that Nrf2 is an important transcription factor for the prevention of hepatocyte death during I/R. Nrf2 deficiency aggravated oxidative stress, inflammatory response, and liver damage. Preactivation of Nrf2 before ischemia significantly suppressed oxidative stress, TNF α induction, as well as the extent of liver damage caused by I/R. Development of specific and effective Nrf2 activator would provide a distinct insight into the strategy for liver protection against I/R injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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A

Figure 1. Nrf2-deficient mice are susceptible to hepatic I/R injury and 15d-PGJ2 has a protective effect in an Nrf2-dependent manner

(A) Serum concentrations of AST/ ALT were measured 6 hours after reperfusion. Data represent means $\pm$ SEM. *, $p < 0.05$ vs. vehicle treated Nrf2^{+/+} mice. n=10 per group. (B) Nrf2 nuclear translocation in primary hepatocytes isolated from Nrf2^{+/+} mice was determined by immunofluorescence. Original magnification was $\times 100$. The figures are representative of 4 independent experiments.

A

B

C

Figure 2. Oncotic necrosis caused by hepatic I/R is exacerbated in Nrf2^{-/-} mice, but it is alleviated in 15d-PGJ2-treated Nrf2^{+/+} mice

Six hours after reperfusion, liver tissues were harvested. (A) Liver sections were stained with hematoxylin/eosin. The figures are representative of 6 independent experiments. Original magnification was $\times 400$ (inset, $\times 800$). (B) DNA fragmentation was assessed by TUNEL staining. (C) TUNEL-positive hepatocytes were counted in 5 high-power ($\times 400$) fields. The numbers were expressed as the percentage of TUNEL-positive hepatocytes of the total hepatocytes in the fields. Data represent means $\pm$ SEM. *, $p < 0.05$ vs. vehicle-treated Nrf2^{+/+} mice. N.S., not significant. $n = 6$ per group.

E

Figure 3. The expression of antioxidant genes is reduced in Nrf2^{-/-} mice, and it is augmented in 15d-PGJ2-treated Nrf2^{+/+} mice

Total hepatic RNA and protein were extracted before ischemia and after I/R. (A) GSTm1, (B) NQO1, (C) GCLc, and (D) HO-1 mRNA expressions were quantified by real-time RT-PCR. The expression was normalized as a ratio to GAPDH mRNA as a housekeeping gene. Data represent mean $\pm$ SEM. (E) The protein expressions of NQO1 and GCLc were evaluated by Western blot analysis. The figure shown is representative of 4 independent experiments. (*, $p < 0.05$ vs. vehicle-treated Nrf2^{+/+} control mice. #, $p < 0.05$ vs. vehicle-treated Nrf2^{+/+} I/R mice. N.S., not significant. $n = 5$ per group).

Figure 4. Oxidative stress and induction of TNF- α are aggravated in Nrf2^{-/-} mice, but these are alleviated by 15d-PGJ2 only in Nrf2^{+/+} mice

Before ischemia and 3 to 6 hours after reperfusion, (A) GSH/GSSG ratio, (B), MDA contents, and (C) TNF- α mRNA in the liver tissues were measured. Data represent mean $\pm$ SEM. (*, $p < 0.05$ vs. vehicle-treated Nrf2^{+/+} control mice. #, $p < 0.05$ vs. vehicle-treated Nrf2^{+/+} I/R mice. N.S., not significant. $n = 4$ to 5 per group).

A

B

Figure 5. The mRNA expression of PPAR γ is Nrf2-dependent, and GW9662 suppresses the upregulation of PPAR γ target genes induced by 15d-PGJ2

Total hepatic RNA was extracted before ischemia. (A) PPAR γ and (B) CD36, AGCA1, ABCG1, and LXR α mRNA expressions were quantified by real-time RT-PCR. Data represent mean $\pm$ SEM. (*, $p < 0.05$ vs. vehicle-treated *Nrf2*^{+/+} control mice. #, $p < 0.05$ vs. 15d-PGJ2-treated *Nrf2*^{+/+} mice. N.S, not significant. n=6 per group).