Available online at www.sciencedirect.com

SciVerse ScienceDirect

Journal of Nutritional Biochemistry 23 (2012) 1249–1255

Journal of Nutritional Biochemistry

Protective effects of chlorogenic acid against ischemia/reperfusion injury in rat liver: molecular evidence of its antioxidant and anti-inflammatory properties

Nari Yun, Jung-Woo Kang, Sun-Mee Lee*

School of Pharmacy, Sungkyunkwan University, Suwon, Gyeonggi-do 440-746, South Korea

Received 17 February 2011; received in revised form 20 June 2011; accepted 29 June 2011

Abstract

Hepatic ischemia and reperfusion injury (I/R) is accompanied by excessive reactive oxygen species and resultant sterile inflammation. Chlorogenic acid (CGA), one of the most abundant polyphenols in the human diet, has been shown to exert potent anti-inflammatory, antibacterial and antioxidant activities. Thus, the purpose of the present study was to investigate protective effects of CGA and its molecular mechanisms against hepatic I/R injury. Rats were subjected to 60 min of partial hepatic ischemia followed by 5 h of reperfusion. CGA (2.5, 5 and 10 mg/kg, ip) was administered twice: 10 min prior to ischemia and 10 min before reperfusion. CGA treatment resulted in marked improvement of hepatic function and histology, and suppressed oxidative stress, as indicated by hepatic lipid peroxidation and glutathione level. Levels of serum tumor necrosis factor-α, inducible nitric oxide synthase and cyclooxygenase-2 protein and mRNA expressions were up-regulated after I/R; these effects were attenuated by CGA. Immunoblot results showed that CGA reduced I/R-induced toll-like receptor 4 overexpression, nuclear translocation of nuclear factor kappa B and interferon regulatory factor-1, high-mobility group box-1 release into extracellular milieu, and enhanced heme oxygenase-1 expression and nuclear translocation of nuclear factor erythroid 2-related factor 2. Our results suggest that CGA alleviates I/R-induced liver injury and that this protection is likely due to inhibition of inflammatory response and enhancement of antioxidant defense systems. Therefore, CGA might have potential as an agent for use in clinical treatment of hepatic I/R injury.

Keywords: Chlorogenic acid; Hepatic ischemia/reperfusion; High-mobility group box 1; Heme oxygenase-1

1. Introduction

Hepatic ischemia and reperfusion (I/R) injury is commonly encountered in a variety of clinical settings, such as trauma, shock, liver transplantation and electric liver resection [1]. The mechanisms underlying hepatic I/R injury are highly complex; however, it is now becoming clear that excessive production of reactive oxygen species (ROS) during reperfusion causes cellular damage by direct attack on various cellular molecules and indirectly by promoting synthesis of proinflammatory mediators [2]. Excessive ROS during the initial phase of reperfusion has recently been shown to act as a signaling molecule inducing release of endogenous damage-associated molecular patterns (DAMPs), which are responsible for propagation of the inflammatory response and further hepatic damage [3,4]. DAMPs, endogenous danger signals, are released during ischemia or tissue injury and are known to interact with certain pattern recognition receptors (PRRs), especially toll-like receptors (TLRs), to activate the innate immune system [5].

High-mobility group box 1 (HMGB1), which was initially identified as a chromatin-bounding protein in the nucleus, has been shown to act as an extracellular signaling molecule and as a DAMP [6]. Release of HMGB1 results in activation of innate immune cells, possibly by engaging with PRRs, such as TLR2, 4, and receptor for advanced glycation end products [7]. In the liver, HMGB1 release was increased during hepatic I/R as early as 1 h after reperfusion, and neutralizing antibody to HMGB1 decreased production of inflammatory mediators, including tumor necrosis factor (TNF), interleukin (IL)-6 and inducible nitric oxide synthase (iNOS) [3]. In isolated hepatocytes, hypoxia-induced HMGB1 release was promoted by TLR4-dependent ROS production [8]. Recent investigation has also demonstrated that activation of interferon regulatory factor-1 (IRF-1), a transcription factor originally identified as a regulator of interferon-α and β, contributes to HMGB1 release from hepatocytes during hepatic I/R [9]. Pardo et al. [10] reported on regulation of endogenous antioxidant defense against hepatic I/R injury by DAMPs.

Heme oxygnase-1 (HO-1), a rate-limiting enzyme of heme degradation, is an endogenous, cytoprotective enzyme that is up-regulated under conditions of oxidative stress [11]. This highly inducible enzyme is up-regulated primarily at a transcriptional level through binding of redox-sensitive transcription factors, such as activator protein-1 (AP-1) and nuclear factor erythroid 2-related factor 2 (Nrf2), to antioxidant redox elements (ARE) located in the promoter of the *ho-1* gene [12]. Overexpressed HO-1 has been shown to exert potent antioxidant, anti-inflammatory and antiapoptotic functions against I/R-induced hepatic injury [13,14]. Recent studies have shown that local induction of HO-1 attenuated TLR overexpression and HMGB1 release during hepatic I/R [15,16].

* Corresponding author. Tel.: +82 31 290 7712; fax: +82 31 292 8800.
E-mail address: sunmee@skku.edu (S.-M. Lee).

0955-2863/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2011.06.018

Table 1
RT-PCR primers used in study and the amplified product length

Gene	Accession number	Primer sequences (5′-3′)	Product length (bp)
TNF-α	NM_012675	Sense: GTA GCC CAC GTC GTA GCA AA Antisense: CCC TTC TCC AGC TGG AAG AC	347
iNOS	NM_012589	Sense: TTC TTT GCT TCT GTG CTT AAT GCG Antisense: GTT GTT GCT GAA CTT CCA ATC GT	3793
COX-2	NM_011198	Sense: ACT CAC TCA GTT TGT TGA GTC ATT C Antisense: TTT GAT TAG TAC TGT AGG GTT AAT G	582
HO-1	NW_047532	Sense: AAG GAG TTT CAC ATC CTT GCA Antisense: ATG TTG AGC AGG AAG GCG GTC	1395
β-*actin*	NM_031144	Sense: TTG TAA CCA ACT GGG ACG ATA TGG Antisense: GAT CTT GAT CTT CAT GGT GCT AG	764

Chlorogenic acid (CGA), formed by esterification of caffeic and quinic acids, is one of the most abundant polyphenols in nature [17]. Due to its easy accessibility in the human diet, a large body of studies has been conducted to investigate its biological functions, including anti-inflammatory, antibacterial, antioxidant and anticarcinogenic activities in several animal models of disease [18,19]. In A549 human cancer cells, CGA induced up-regulation of cellular antioxidant enzyme and suppressed ROS-mediated nuclear factor kappa B (NF-κB), AP-1 and mitogen-activated protein kinase activations [20]. In addition, recent evidence has shown that CGA has an antihepatotoxic effect on LPS-challenged mice by suppression of the mRNA levels of TLR4, TNF-α and the NF-κB p65 subunit [21].

Therefore, this study was designed to investigate the protective effects of CGA against hepatic damage induced by I/R and its molecular mechanisms of anti-inflammatory and antioxidant functions.

2. Methods and materials

2.1. Liver warm ischemia and reperfusion procedure

Male Sprague–Dawley rats (body weight 270–300 g, Orient bio, Inc., Gapyeong, Korea) were fasted for 18 h, but allowed free access to tap water. All animal protocols were approved by the Animal Care Committee of Sungkyunkwan University and were performed in accordance with the guidelines of the National Institutes of Health. Under ketamine (60 mg/kg, ip) and xylazine (8 mg/kg, ip) anesthesia, a midline laparotomy was performed. Using an operating microscope, the liver hilum was exposed, and portal structures to the left and median lobes were occluded with a microvascular clamp (Biomedical Research Instruments, Inc., Rockville, MD, USA) for 60 min; reperfusion was initiated by removal of the clamp. At 5 h after reperfusion, blood was obtained from abdominal aorta, and the liver tissues were stored at −75°C for later analysis, except for the part in the left lobe, which was used for histological analysis.

2.2. Administration of CGA

CGA was dissolved in saline (vehicle). Rats were administered twice at doses of 2.5, 5 and 10 mg/kg of CGA (ip) at 10 min before ischemia and 10 min before reperfusion. The dose and the injection time of CGA were based on previous reports [22] and our preliminary studies. Rats were randomly divided into four groups: (1) vehicle-treated sham, (2) CGA-treated sham, (3) vehicle-treated ischemic (I/R) and (4) CGA-treated I/R. No differences were observed in any of the parameters between vehicle-treated and CGA-treated rats in the sham groups; therefore, the results of groups 1 and 2 were pooled and were referred to as sham.

2.3. Histological analysis

Formalin-fixed samples were embedded in paraffin and cut into 5-μm sections. Tissues were stained with hematoxylin and eosin (H&E), and slides were assessed for inflammation and tissue damage using Olympus microscopy (OLYMPUS OPTICAL Co., Tokyo, Japan) at a 200× magnification.

2.4. Serum alanine aminotransferase activity

Serum alanine aminotransferase (ALT) levels were measured by the standard spectrophotometric procedure using a ChemiLab ALT assay kit (IVDLab Co., Ltd., Uiwang, Korea).

2.5. Hepatic lipid peroxidation and glutathione content

The steady-state level of malondialdehyde (MDA), the end-product of lipid peroxidation, was analyzed in liver homogenates by spectrophotometric measurement of the level of thiobarbituric acid-reactive substances at a wavelength of 535 nm according to the method described by Buege and Aust [23] using 1,1,3,3,-tetraethoxypropane as the standard. Total glutathione in the liver homogenate was determined spectrophotometrically at a wavelength of 412 nm using yeast glutathione reductase, 5,5′-dithio-bis(2-nitrobenzoic acid) and NADPH according to the method reported by Tietze [24]. The oxidized glutathione (GSSG) level was measured using the same method in the presence of 2-vinylpyridine, and the reduced glutathione (GSH) level was determined from the difference between the total glutathione and GSSG levels.

2.6. Protein extraction of whole liver tissue

Isolated liver tissue was homogenized in PRO-PREP Protein Extraction Solution (iNtRON Biotechnology Inc., Seongnam, Korea) in a microcentrifuge tube. After standing in a cold ice bath for a period of 30 min for cell lysis, the whole homogenate was centrifuged at 13,000*g* for 5 min. The supernatant was collected, and the protein concentrations of the whole homogenates were determined using the BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA).

2.7. Isolation of cytosolic and nuclear proteins

NE-PER (Pierce Biotechnology, Rockford, IL, USA) was used for extraction of nuclear and cytosolic fractions according to the manufacturer's instructions. Briefly, isolated liver tissue was homogenized in cold Cytoplasmic Extraction Reagent (CER) 1 (with protease inhibitor cocktail set III; Calbiochem, La Jolla, CA, USA). After incubation for 10 min, CER2 was added to break down the cytoplasmic membrane. After

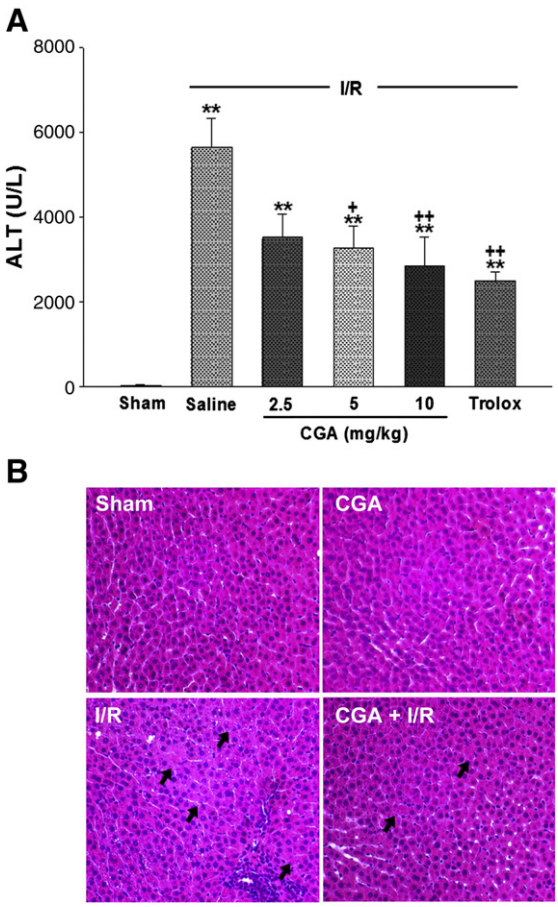

Fig. 1. Effect of CGA on serum ALT activity (A) and histological features of liver sections stained with H&E (B) at 5 h after reperfusion. Typical images were chosen from each experimental group (original magnification×200). Sham and CGA: normal lobular architecture and cell structure; I/R: hepatocellular necrosis with extensive inflammation and inflammatory cell infiltration; CGA+I/R: mild hepatocellular necrosis and inflammatory infiltration. Arrows indicate necrotic hepatocyte and inflammatory cell infiltration. Data represent means±S.E.M., n=8–10 rats per group. **Significantly different (P<.01) from sham. $^{+,++}$Significantly different (P<.05, P<.01) from I/R.

Table 2
Effects of CGA on GSH and MDA levels following hepatic I/R

Group	MDA (nmol/mg protein)	GSH (μmol/g liver)
Sham	1.4±0.1	5.1±0.2
I/R	7.7±0.8**	2.3±0.5**
CGA+I/R	1.5±0.1++	4.4±0.7*,+

Results are presented as mean±S.E.M. of 8–10 animals per group. CGA at a dose of 10 mg/kg was administered intraperitoneally 10 min prior to ischemia and 10 min before reperfusion. Levels of GSH and MDA were determined at 5 h after reperfusion.
*,**Significantly different (P<.05, P<.01) from sham.
+,++Significantly different (P<.05, P<.01) from I/R.

centrifuging (16 000 *g* for 5 min), the cytoplasmic extract was collected. Nuclear Extract Reagent with protease inhibitor cocktail set III (Calbiochem) was added to the remaining nuclear pellet, and after a 40-min incubation and centrifugation (16,000*g* for 10 min), the nuclear extract was harvested. Protein concentrations were determined using the BCA Protein Assay kit (Pierce Biotechnology).

2.8. Serum preparation for HMGB1 analysis

Serum samples were filtered and concentrated through Centricon YM-100 and YM-10 (Millipore, Billerica, MA, USA) with fixed angle (35°C), 7500 *g* for 15 min, 4°C, respectively. The concentrated samples were then subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE).

2.9. Immunoblots

Protein samples were loaded on 10%–15% polyacrylamide gels and were then separated by SDS/PAGE and transferred to polyvinylidene fluoride membranes (Millipore) using the Semi-Dry Trans-Blot Cell (Biorad Laboratories, Hercules, CA, USA). After transfer, the membranes were washed with 0.1% Tween-20 in 1× Tris-buffered saline (TBS/T) and blocked for 1 h at room temperature with 5% (w/v) skim milk powder in TBS/T. The blots were then incubated overnight at 4°C with primary antibodies. After washing three times for 5 min each in TBS/T, the membranes were incubated with appropriate secondary antibodies for 1 h at room temperature and detected using an ECL detection system (iNtRON Biotechnology Inc.) according to the manufacturer's instructions. ImageQuant TL software (Amersham Biosciences/GE Healthcare, Piscataway, NJ, USA) was used for densitometric evaluation of visualized immunoreactive bands. Primary antibodies against IRF-1 (1:200 dilution; Santa Biotechnology, Santa Cruz, CA, USA), HMGB1 (1:1000 dilution; Abcam, Cambridge, UK), TLR2 and 4 (1:1000 dilution; Santa Cruz Biotechnology), HO-1 (1:1000 dilution; StressGene Biotechnology, Victoria, Canada), Nrf2 (1:500 dilution; Santa Cruz Biotechnology), NF-κB/p65 (1:1000 dilution; Santa Cruz Biotechnology), c-Jun p39 phosphorylated on serine-63 (1:500 dilution; Santa Cruz Biotechnology), iNOS (1:1000 dilution; Transduction Laboratory, Lexington, KY, USA) and cyclooxygenase-2 (COX-2) (1:500 dilution; Cayman Chemical, Ann Arbor, MI, USA) were used, and the signals were standardized to β-actin (1:2000 dilution; Sigma Chemical Co.) for whole lysate and lamin B1 (1:2500 dilution; Abcam) for the nuclear fraction.

2.10. Enzyme-linked immunosorbent assay (ELISA)

A commercial TNF-α ELISA kit (BD Biosciences Co., CA, USA) was used for quantification of the serum TNF-α level.

2.11. Total RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted, and the first-strand cDNA was synthesized by reverse transcription of the total RNA using the oligo(dT) primer and SuperScript II RNase H-Reverse Transcriptase (Invitrogen Tech-Line, Carlsbad, CA, USA). PCR was carried out in a 20-μl reaction volume with a diluted cDNA sample. The final reaction concentrations were as follows: sense and antisense primers, 10 pM; dNTP mix, 250 μM; 10× PCR buffer; and Ex Taq DNA polymerase, 0.5 U/reaction. PCR was performed with an initial denaturation step at 94°C for 5 min and a final extension step at 72°C for 7 min in the GeneAmp 2700 thermocycler (Applied Biosystems, Foster City, CA, USA). Gene-specific primers used are listed in Table 1. Amplification cycling conditions were as follows: 40 cycles at 94°C (30s), 65°C (30s), 72°C (30s) for COX-1; 25 cycles at 94°C (30s), 56°C (30s), 72°C (30s) for β-actin. Following RT-PCR, 10-μl samples of the PCR products were visualized by ultraviolet illumination after electrophoresis through 1.5% agarose gel and ethidium bromide staining. SLP Mylmager (UVP Inc., Upland, CA, USA) and ImageQuant TL (Amersham Biosciences/GE Healthcare) were used for semiquantitative analysis of the intensity of each PCR product.

2.12. Statistical analysis

The overall significance of the results was examined using one-way analysis of variance. The difference between the groups was considered statistically significant at P<.05, with the appropriate Bonferroni correction made for multiple comparisons. The results are presented as the mean±S.E.M.

3. Results

3.1. Serum ALT activity and histological analysis

In the sham-operated animals, serum ALT activity averaged 27.6±11.6 U/L. Compared to sham-operated animals, rats subjected to I/R showed a dramatic increase in serum ALT level (5648.5±684.5 U/L, P<.01). Treatment with 2.5 mg/kg CGA did not affect serum ALT level; however, doses of 5 and 10 mg/kg CGA induced marked attenuation of elevated ALT levels (3275.2±545.1 and 2840.4±688.8 U/L, P<.05 and P<.01, respectively; Fig. 1A). Thus, CGA at 10 mg/kg was selected as the optimal effective dose for evaluation of the histology and the molecular mechanisms of CGA against I/R-induced hepatic injury. While normal liver lobular architecture and cell structure were observed in sham-operated animals, liver sections from rats undergoing I/R showed multiple and extensive areas of portal inflammation and hepatocyte necrosis, which were randomly distributed throughout the parenchyma, as well as

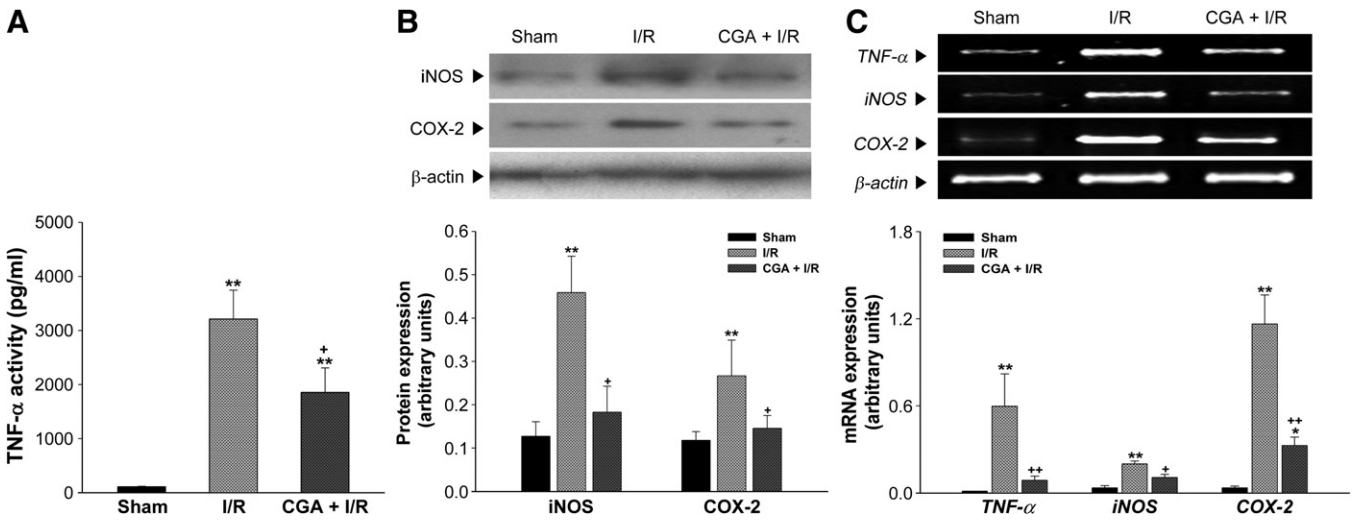

Fig. 2. Effect of CGA on serum TNF-α level (A), protein expression of iNOS and COX-2 (B), and mRNA expression of TNF-α, iNOS, and COX-2 (C) at 5 h after reperfusion. Data represent means±S.E.M., n=8–10 rats per group. Immunoblot and PCR shown are representative of three experiments with similar results. *,**Significantly different (P<.05, P<.01) from sham. +,++Significantly different (P<.05, P<.01) from I/R.

a moderate increase in inflammatory cell infiltration. These pathological changes were inhibited by CGA treatment (Fig. 1B).

3.2. Hepatic lipid peroxidation and glutathione contents

As shown in Table 2, hepatic MDA level in liver tissues was 1.4±0.1 nmol/mg protein in sham-operated animals. After 5 h of reperfusion, the MDA level showed a significant increase to 7.7±0.8 nmol/mg protein. However, this elevation in MDA level was significantly attenuated by CGA treatment (1.5±0.1 nmol/mg protein, $P<.05$). The GSH content in sham-operated animals was 5.1±0.2 μmol/g liver. In contrast to an increased level of MDA in ischemic liver, GSH content showed a marked decrease to 2.3±0.5 μmol/g liver at 5 h after reperfusion, and CGA treatment resulted in restoration of this decrease (4.4±0.7 μmol/g liver, $P<.05$).

3.3. Inflammatory cytokine levels

In the sham-operated animals, serum TNF-α level was at the basal level (108.2±10.2 pg/ml). After 5 h of reperfusion, the serum TNF-α level showed a significant increase to 3210.7±535.5 pg/ml, and this increase was markedly attenuated by CGA treatment (1855.0±454.5 pg/ml, $P<.05$, Fig. 2A). In addition, I/R resulted in a dramatic increase of iNOS and COX-2 protein expression by 3.6-fold and 2.1-fold over basal levels, respectively, and these increases were attenuated by CGA treatment ($P<.05$, Fig. 2B). Results shown in Fig. 2C demonstrated the mRNA levels of TNF-α, iNOS and COX-2, while rats subjected to I/R showed significant increases in TNF-α, iNOS, and COX-2 mRNA levels by 45.6-fold, 5.5-fold and 33.2-fold over basal levels, respectively; CGA attenuated these increases by 85.3%, 47.4% and 72% of I/R groups, respectively.

3.4. HO-1 protein and mRNA expression and nuclear translocation of Nrf2, NF-κB and p-c-Jun

HO-1 protein expression in rats subjected to I/R was significantly increased by 2.6-fold over that of sham-operated animals ($P<.01$). CGA treatment augmented HO-1 protein expression, approximately 1.3 times that of the I/R group ($P<.05$). Similarly, the mRNA levels of *HO-1* determined in rats undergoing I/R showed a marked increase compared to sham-operated animals, and CGA treatment resulted in further increase of *HO-1* mRNA levels (Fig. 3A). Immunoblots shown in Fig. 3B demonstrated nuclear translocation of Nrf2, NF-κB/p65 and activated AP-1/c-Jun (p-c-Jun). Following 5 h of reperfusion, ischemic liver showed marked increase in nuclear translocation of transcription factors, approximately 3.3-fold for Nrf2 ($P<.05$), 5.4-fold for NF-κB/p65 ($P<.05$) and 4.8-fold for p-c-Jun ($P<.01$) over basal levels. CGA did not alter the elevated nuclear protein expression of p-c-Jun; however, it induced marked augmentation of nuclear translocation of Nrf2 approximately 1.5 times that of the I/R group ($P<.05$). In contrast, CGA treatment inhibited nuclear translocation of NF-κB/p65 by 62.4% of that of the I/R group ($P<.05$).

3.5. HMGB1 translocation, serum release and nuclear translocation of IRF-1

Fig. 4A shows that HMGB1 translocation, the ratio of band intensity of cytosolic and nuclear HMGB1, in sham-operated rats remained at basal levels. Reperfusion resulted in a dramatic increase in HMGB1 translocation approximately 2.3 times that of the sham group, and CGA treatment significantly attenuated this increase by 25.8% ($P<.05$). Ensuing data in Fig. 4B illustrate HMGB1 release into the extracellular milieu. In the sham-operated animals, serum HMGB1 release was barely detectable. I/R resulted in a

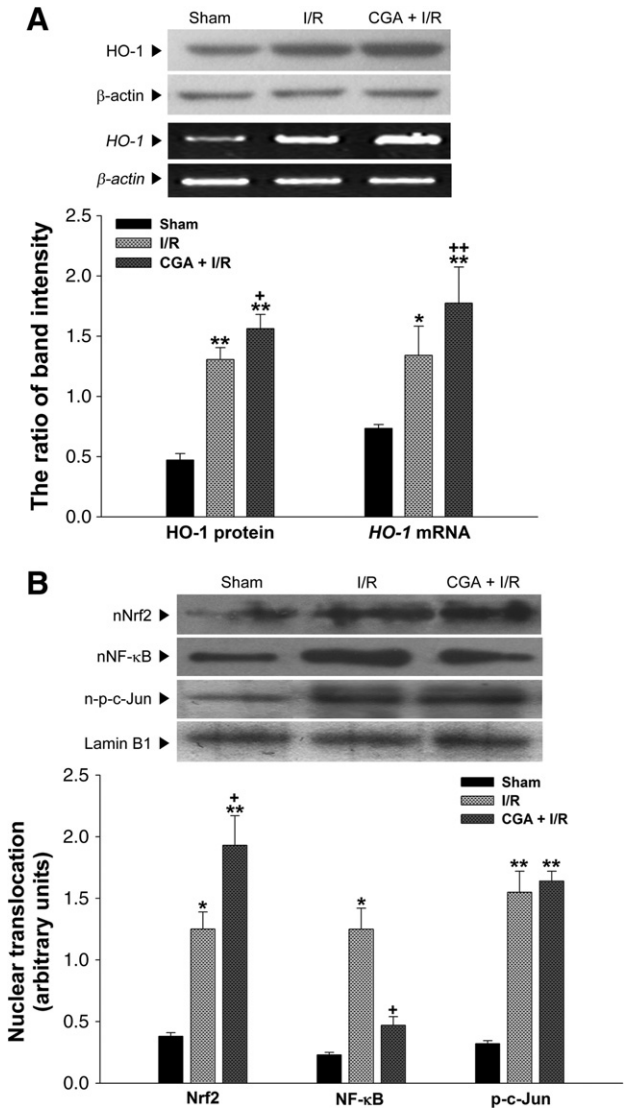

Fig. 3. Effect of CGA on protein and mRNA expression of HO-1 (A) and nuclear translocation of Nrf2, NF-κB, and p-c-Jun (B) at 5 h after reperfusion. Data represent means±S.E.M., $n=8$–10 rats per group. Immunoblot and PCR shown are representative of three experiments with similar results. *,**Significantly different ($P<.05$, $P<.01$) from sham. $^{+,++}$Significantly different ($P<.05$, $P<.01$) from I/R.

marked increase of serum HMGB1 release, about 6.5-fold over that of the sham group ($P<.01$); however, CGA treatment resulted in significant attenuation of this increase by 67.7% ($P<.01$). Nuclear translocation of IRF-1 in sham-operated animals was quite low, while ischemic liver showed marked increase in approximately 1.4-fold over that of the sham group ($P<.01$). CGA treatment inhibited this increase by 76.2% ($P<.05$).

3.6. TLR4 and TLR2 protein expression

TLR4 protein expression was markedly increased, approximately 3.3 times that of the sham group ($P<.05$), and CGA pretreatment attenuated overexpressed TLR4 protein by 30.0% of that of the I/R group ($P<.01$). TLR2 protein expression in rats subjected to I/R showed only a tendency to increase compared to that of sham-operated animals, and CGA did not affect this increase (Fig. 5).

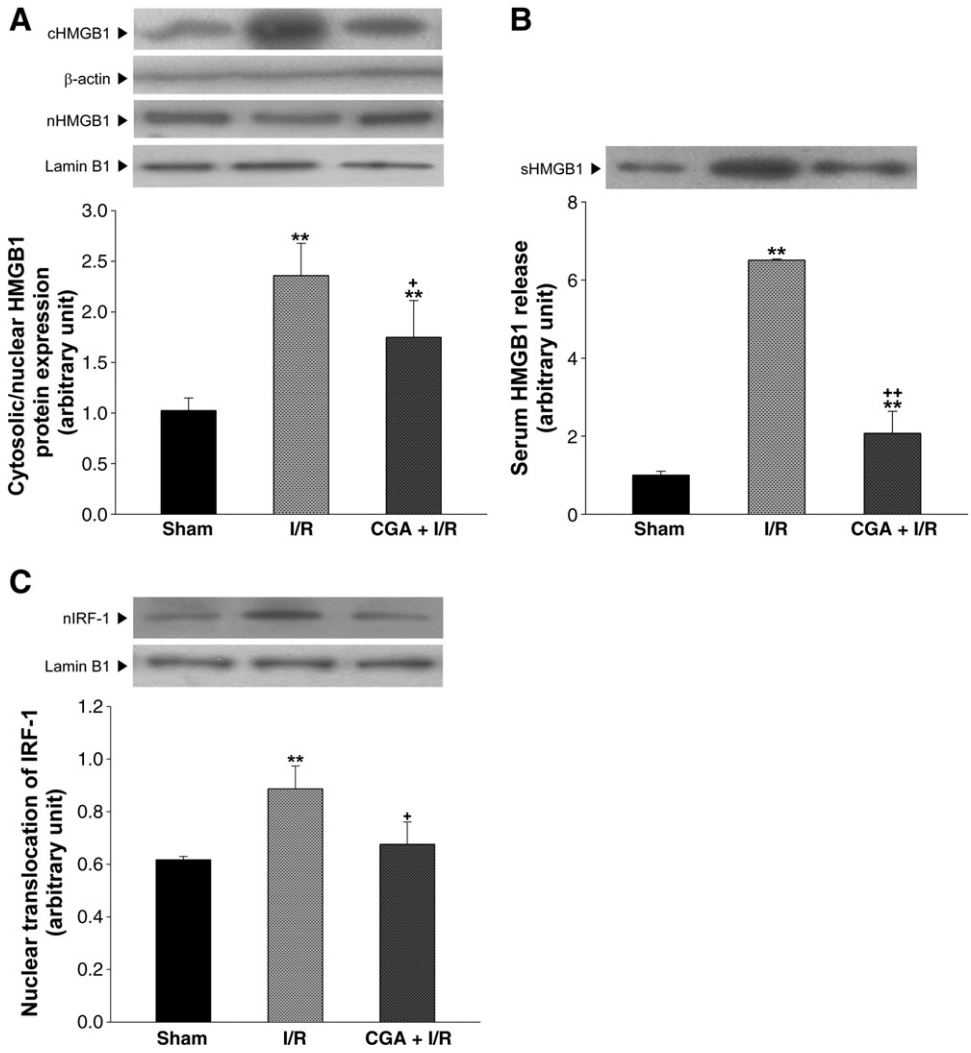

Fig. 4. Effect of CGA on cytosolic translocation (A) and serum release (B) of HMGB1 at 5 h after reperfusion. Data represent means±S.E.M., n=8–10 rats per group. Immunoblot shown is a representative of three experiments with similar results. Serum HMGB1 protein level of sham group was arbitrarily set at 1.0. **Significantly different (P<.01) from sham. $^{+,++}$Significantly different (P<.05, P<.01) from I/R.

4. Discussion

ROS produced during reperfusion is generally accepted as playing a central role in I/R injury by direct attack on a vast array of cellular molecules; recent evidence has shown that it also directly activates the innate immune system by promoting release of DAMPs [25]. Thus, limiting oxidative damage in the initial phase of reperfusion is likely essential to prevention of detrimental consequences of overall inflammatory response during I/R.

CGA is one of the most abundant polyphenols in the human diet and has been shown to exert potent antioxidant and anti-inflammatory activities [18]. CGA has been shown to act as a scavenger of superoxide radicals, hydroxyl radicals and peroxynitrite in a concentration-dependent manner *in vitro* and also shows antioxidant activities against indomethacin-induced gastric mucosal damage *in vivo* [26,27]. Based on this, we observed a protective effect of CGA against hepatic I/R injury and molecular evidence for hepatoprotection of CGA. In our results, serum ALT level showed a marked increase in rats subjected to I/R, and this increase was significantly attenuated by CGA. These results were also supported by histological observations; while liver obtained from rats undergoing I/R showed multiple and extensive areas of hepatocyte necrosis randomly distributed throughout the parenchyma, only mild centrizonal necrosis and Kupffer cell hyperplasia were observed in the CGA-treated group. To further explore the possibility of CGA as a potential therapeutic strategy against hepatic I/R, CGA was administered only at 10 min prior to reperfusion, without administration before ischemia, at a dose of 10 mg/kg. Serum ALT level in CGA posttreated rat was significantly reduced compared to that of ischemic animals, and the protection seen in the postischemic-treated one was less than that in preischemic treatment (data not shown). The present results demonstrate that treatment of CGA improves hepatic function after I/R.

HMGB1 is a ubiquitous DNA-binding protein that is either passively secreted by necrotic cells or actively secreted by immunocompetent cells and has received particular attention as fulfilling the functions of a DAMP [5,28]. Indeed, a large body of evidence has shown that a substantial amount of HMGB1 is released prior to production of classical cytokines, such as TNF-α and IL-1β, even in the absence of infection [6]. Neutralizing antibodies against HMGB1 ameliorated the inflammatory response and organ damage in several models of sterile injury, including hemorrhagic shock [29] and femur fracture [30], as well as hepatic I/R [3]. More recent studies have demonstrated how HMGB1 can be actively released from damaged hepatocytes during I/R and revealed that nuclear translocation of IRF-1 not only mediates liver damage during I/R injury, but also is responsible for HMGB1

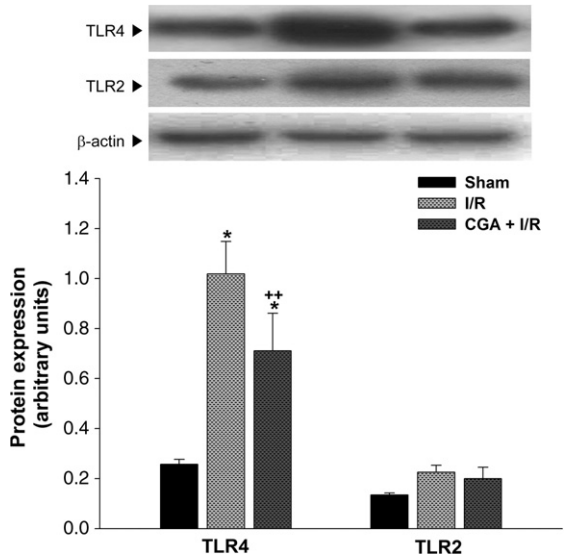

Fig. 5. Effect of CGA on protein expression of TLR4 and TLR2 at 5 h after reperfusion. Data represent means±S.E.M., n=8–10 rats per group. Immunoblot shown is representative of three experiments with similar results. *Significantly different (P<.05) from sham. ++Significantly different (P<.01) from I/R.

release from hepatocytes [9,31]. In the present study, rats subjected to hepatic I/R showed significant increase in serum HMGB1 release after reperfusion; however, CGA significantly attenuated this increase. A similar result was observed in cytosolic translocation of HMGB1, which suggests that CGA suppresses HMGB1 translocation and release into the extracellular milieu. Furthermore, rats undergoing hepatic I/R showed a dramatic increase in nuclear translocation of IRF-1, which was attenuated by CGA.

TLRs are a family of PRRs that are activated by specific components of microbes referred to as pathogen-associated molecular pattern molecules and certain other molecules, such as DAMPs [32]. In recent years, emerging data have shown involvement of TLRs and their interaction with endogenous TLR ligands in initiation of inflammatory response seen in hepatic I/R [4,32], and HMGB1 is known to stimulate TLR4 signaling in this process [3]. During hepatic I/R, HMGB1 released in the early phase of reperfusion exacerbated hepatic damage through TLR4-dependent signaling [8]. Meanwhile, some reports have demonstrated the role of TLR2 in progression of hepatic I/R injury, showing less hepatic damage after I/R when TLR2 expression on Kupffer cells decreased [33]. Also, the interaction of HMGB1 with TLR2 cannot be excluded since murine macrophage cell lines transfected with dominant-negative constructs of TLR2 showed decreased activation on stimulation with HMGB1 [34]. In our results, rats undergoing I/R showed considerably higher TLR4 expression compared to that of sham animals, while TLR2 expression showed only a tendency to increase. Although CGA did not alter TLR2 expression, it induced marked attenuation of TLR4 overexpression.

HO-1 has generally been regarded as an adaptive cellular response against oxidative stress [35] and is thought to play a crucial role in the protection of the liver against hepatic I/R injury through its antioxidant, antiapoptotic and anti-inflammatory properties [12,36]. More recently, data obtained from HO-1 knockout mice and a human case of genetic HO-1 deficiency have emphasized its major immunomodulatory roles [37,38]. Overexpression of HO-1 attenuated hepatic I/R injury by down-regulation of the type-1 interferon pathway, downstream of TLR4 [39]. In another report with hepatic I/R, TLR4 protein expression decreased on induction of HO-1 [25]. Furthermore, in our recent report on hepatic I/R, induction of HO-1 by hemin resulted in significantly decreased HMGB1 release, whereas blockage of HO-1 expression with ZnPP resulted in marked augmentation of HMGB1 release [16]. Tsoyi et al. [40] demonstrated that HO-1-derived carbon monoxide reduces HMGB1 release in a lipopolysaccharide-induced or cecal ligation puncture-induced animal model of sepsis. In our study, mRNA levels and protein expression of HO-1 were markedly induced by I/R, and CGA augmented these increases in both mRNA and protein levels. Therefore, CGA may largely regulate HO-1 production at the transcriptional level.

Transcriptional regulation of the *ho-1* gene involves a select set of redox-sensitive transcription factors, such as AP-1, which is composed of structurally and functionally related members of the Jun and Fos and activating transcription factor protein families, and Nrf2 [12]. Translocation of these transcription factors into the nucleus leads to enhanced production of heme-containing enzymes, such as iNOS and HO-1, which further enhance production of endogenous antioxidants, like GSH and NO. However, excessive production of NO following increased iNOS expression generates $ONOO^-$ in the process of scavenging $O2^-$. This $ONOO^-$ can then rapidly oxidize GSH, causing nitrosative stress, and, in response, activation of redox-sensitive transcription factors can be further augmented [41]. Recent evidence has offered strong support for the importance of Nrf2 in stress-dependent HO-1 induction. Although various functional AP-1 sites have been identified in promoter regions of rat and human *ho-1* genes, AP-1-dependent HO-1 gene expression appears to involve synergistic cooperation of AP-1 with other transcription factors such as Nrf2 [42]. Upon activation, Nrf2 enters the nucleus where it binds to the ARE in the HO-1 promoter region to trigger gene expression [43]. HO-1 induction via Nrf2 translocation was found to protect the kidney from remote organ injury after warm hepatic I/R [44]. Furthermore, activation of Nrf2 translocation in A549 human cancer cells by CGA has been demonstrated, and this was associated with anticarcinogenic effects [20]. In our study, rats subjected to I/R showed a dramatic increase in Nrf2 translocation, and CGA treatment augmented the increase. Although translocation of p-c-Jun, the predominant active form of AP-1 in postreperfused liver, also increased in rats undergoing I/R, CGA treatment did not alter the increase of p-c-Jun translocation. Notably, overexpression of iNOS and decreased GSH content by I/R were also significantly attenuated by CGA treatment. Overall, these data indicate that CGA may reduce oxidative/nitrosative stress occurring during I/R by enhancement of Nrf2 translocation and subsequent induction of HO-1.

Activation of the NF-κB signaling pathway is central to the pathophysiology of the inflammatory response under several experimental conditions, including hepatic warm I/R [45]. The functional importance of NF-κB in inflammation is based on its ability to regulate transcription of multiple inflammatory genes, including TNF-α, IL-1β, iNOS and COX-2 [46,47], which are all known as classical proinflammatory mediators produced during the early phase of reperfusion. The importance of NF-κB signaling in hepatic I/R also lies in its interconnection with other redox-sensitive transcription factors. Recent study has demonstrated that disruption of Nrf2 resulted in enhanced up-regulation of NF-κB activity and proinflammatory cytokines in the brain after traumatic brain injury [48]. In addition, Nrf2-activating agent LCY-2-CHO inhibited IL-1β-induced iNOS and COX-2 expression, with concomitant inhibition of NF-κB activation [49]. Our results showed marked increase in NF-κB translocation after reperfusion, and CGA attenuated this increase. Increased TNF-α activity, and mRNA and protein levels of iNOS and COX-2 after reperfusion were also significantly attenuated by CGA treatment. Thus, CGA might exert its anti-inflammatory effect by inhibiting NF-κB translocation.

In summary, our findings suggest that CGA protects against hepatic I/R-induced liver injury. The mechanism of action of CGA appears to involve its ability to inhibit HMGB1 release into the extracellular milieu, TLR4 overexpression, nuclear translocation of

NF-κB and IRF-1, and proinflammatory mediator expression, and to induce the Nrf2/HO-1 pathway. Thus, we propose that CGA is a potential therapeutic medication for prevention of hepatic I/R injury in clinical settings.

Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0028646).

References

[1] Cardinal J, Pan P, Tsung A. Protective role of cisplatin in ischemic liver injury through induction of autophagy. Autophagy 2009;5:1211–2.
[2] Arii S, Teramoto K, Kawamura T. Current progress in the understanding of and therapeutic strategies for ischemia and reperfusion injury of the liver. J Hepatobiliary Pancreat Surg 2003;10:189–94.
[3] Tsung A, Sahai R, Tanaka H, Nakao A, Fink MP, Lotze MT, et al. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia–reperfusion. J Exp Med 2005;201:1135–43.
[4] Zhai Y, Shen XD, O'Connell R, Gao F, Lassman C, Busuttil RW, et al. Cutting edge: TLR4 activation mediates liver ischemia/reperfusion inflammatory response via IFN regulatory factor 3-dependent MyD88-independent pathway. J Immunol 2004;173:7115–9.
[5] Bianchi ME, Manfredi AA. High-mobility group box 1 (HMGB1) protein at the crossroads between innate and adaptive immunity. Immunol Rev 2007;220: 35–46.
[6] Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. Nat Rev Immunol 2005;5:331–42.
[7] Lotze MT, Zeh HJ, Rubartelli A, Sparvero LJ, Amoscato AA, Washburn NR, et al. The grateful dead: damage-associated molecular pattern molecules and reduction/ oxidation regulate immunity. Immunol Rev 2007;220:60–81.
[8] Tsung A, Klune JR, Zhang X, Jeyabalan G, Cao Z, Peng X, et al. HMGB1 release induced by liver ischemia involves toll-like receptor 4 dependent reactive oxygen species production and calcium-mediated signaling. J Exp Med 2007;204: 2913–23.
[9] Dhupar R, Klune JR, Evankovich J, Cardinal J, Zhang M, Ross M, et al. Interferon regulatory factor 1 mediates acetylation and release of high mobility group box 1 from hepatocytes during murine liver ischemia–reperfusion injury. Shock 2011;35:293–301.
[10] Pardo M, Budick-Harmelin N, Tirosh B, Tirosh O. Antioxidant defense in hepatic ischemia–reperfusion injury is regulated by damage-associated molecular pattern signal molecules. Free Radic Biol Med 2008;45:1073–83.
[11] Amersi F, Buelow R, Kato H, Ke B, Coito AJ, Shen XD, et al. Upregulation of heme oxygenase-1 protects genetically fat Zucker rat livers from ischemia/reperfusion injury. J Clin Invest 1999;104:1631–9.
[12] Paine A, Eiz-Vesper B, Blasczyk R, Immenschuh S. Signaling to heme oxygenase-1 and its anti-inflammatory therapeutic potential. Biochem Pharmacol 2010;80: 1895–903.
[13] Ryter SW, Alam J, Choi AM. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. Physiol Rev 2006;86:583–650.
[14] Tsuchihashi S, Livhits M, Zhai Y, Busuttil RW, Araujo JA, Kupiec-Weglinski JW. Basal rather than induced heme oxygenase-1 levels are crucial in the antioxidant cytoprotection. J Immunol 2006;177:4749–57.
[15] Shen XD, Ke B, Zhai Y, Gao F, Busuttil RW, Cheng G, et al. Toll-like receptor and heme oxygenase-1 signaling in hepatic ischemia/reperfusion injury. Am J Transplant 2005;5:1793–800.
[16] Yun N, Eum HA, Lee SM. Protective role of heme oxygenase-1 against liver damage caused by hepatic ischemia and reperfusion in rats. Antioxid Redox Signal 2010;13:1503–12.
[17] Suzuki A, Yamamoto N, Jokura H, Yamamoto M, Fujii A, Tokimitsu I, et al. Chlorogenic acid attenuates hypertension and improves endothelial function in spontaneously hypertensive rats. J Hypertens 2006;24:1065–73.
[18] Bonita JS, Mandarano M, Shuta D, Vinson J. Coffee and cardiovascular disease: in vitro, cellular, animal, and human studies. Pharmacol Res 2007;55:187–98.
[19] dos Santos MD, Almeida MC, Lopes NP, de Souza GE. Evaluation of the anti-inflammatory, analgesic and antipyretic activities of the natural polyphenol chlorogenic acid. Biol Pharm Bull 2006;29:2236–40.
[20] Feng R, Lu Y, Bowman LL, Qian Y, Castranova V, Ding M. Inhibition of activator protein-1, NF-kappaB, and MAPKs and induction of phase 2 detoxifying enzyme activity by chlorogenic acid. J Biol Chem 2005;280:27888–95.
[21] Xu Y, Chen J, Yu X, Tao W, Jiang F, Yin Z, et al. Protective effects of chlorogenic acid on acute hepatotoxicity induced by lipopolysaccharide in mice. Inflamm Res 2010;59:871–7.
[22] Wu HZ, Luo J, Yin YX, Wei Q. Effects of chlorogenic acid, an active compound activating calcineurin, purified from Flos Lonicerae on macrophage. Acta Pharmacol Sin 2004;25:1685–9.
[23] Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol 1978;52: 302–10.
[24] Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem 1969;27:502–22.
[25] Gill R, Tsung A, Billiar T. Linking oxidative stress to inflammation: toll-like receptors. Free Radic Biol Med 2010;48:1121–32.
[26] Graziani G, D'Argenio G, Tuccillo C, Loguercio C, Ritieni A, Morisco F, et al. Apple polyphenol extracts prevent damage to human gastric epithelial cells in vitro and to rat gastric mucosa in vivo. Gut 2005;54:193–200.
[27] Kono Y, Kobayashi K, Tagawa S, Adachi K, Ueda A, Sawa Y, et al. Antioxidant activity of polyphenolics in diets. Rate constants of reactions of chlorogenic acid and caffeic acid with reactive species of oxygen and nitrogen. Biochim Biophys Acta 1997;1335:335–42.
[28] Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 2002;418:191–5.
[29] Yang R, Harada T, Mollen KP, Prince JM, Levy RM, Englert JA, et al. Anti-HMGB1 neutralizing antibody ameliorates gut barrier dysfunction and improves survival after hemorrhagic shock. Mol Med 2006;12:105–14.
[30] Levy RM, Mollen KP, Prince JM, Kaczorowski DJ, Vallabhaneni R, Liu S, et al. Systemic inflammation and remote organ injury following trauma require HMGB1. Am J Physiol Regul Integr Comp Physiol 2007;293:R1538–44.
[31] Tsung A, Stang MT, Ikeda A, Critchlow ND, Izuishi K, Nakao A, et al. The transcription factor interferon regulatory factor-1 mediates liver damage during ischemia–reperfusion injury. Am J Physiol Gastrointest Liver Physiol 2006;290: G1261–8.
[32] Boros P, Bromberg JS. New cellular and molecular immune pathways in ischemia/reperfusion injury. Am J Transplant 2006;6:652–8.
[33] Zhang JX, Wu HS, Wang H, Zhang JH, Wang Y, Zheng QC. Protection against hepatic ischemia/reperfusion injury via downregulation of toll-like receptor 2 expression by inhibition of Kupffer cell function. World J Gastroenterol 2005;11: 4423–6.
[34] Park JS, Svetkauskaite D, He Q, Kim JY, Strassheim D, Ishizaka A, et al. Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. J Biol Chem 2004;279:7370–7.
[35] Gozzelino R, Jeney V, Soares MP. Mechanisms of cell protection by heme oxygenase-1. Annu Rev Pharmacol Toxicol 2010;50:323–54.
[36] Coito AJ, Buelow R, Shen XD, Amersi F, Moore C, Volk HD, et al. Heme oxygenase-1 gene transfer inhibits inducible nitric oxide synthase expression and protects genetically fat Zucker rat livers from ischemia–reperfusion injury. Transplantation 2002;74:96–102.
[37] Poss KD, Tonegawa S. Reduced stress defense in heme oxygenase 1-deficient cells. Proc Natl Acad Sci U S A 1997;94:10925–30.
[38] Yachie A, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, et al. Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. J Clin Invest 1999;103:129–35.
[39] Tsuchihashi S, Zhai Y, Bo Q, Busuttil RW, Kupiec-Weglinski JW. Heme oxygenase-1 mediated cytoprotection against liver ischemia and reperfusion injury: inhibition of type-1 interferon signaling. Transplantation 2007;83:1628–34.
[40] Tsoyi K, Lee TY, Lee YS, Kim HJ, Seo HG, Lee JH, et al. Heme-oxygenase-1 induction and carbon monoxide-releasing molecule inhibit lipopolysaccharide (LPS)-induced high-mobility group box 1 release in vitro and improve survival of mice in LPS- and cecal ligation and puncture-induced sepsis model in vivo. Mol Pharmacol 2009;76:173–82.
[41] Radi R, Beckman JS, Bush KM, Freeman BA. Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. J Biol Chem 1991;266: 4244–50.
[42] Alam J, Stewart D, Touchard C, Boinapally S, Choi AM, Cook JL. Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. J Biol Chem 1999;274:26071–8.
[43] Alam J, Cook JL. Transcriptional regulation of the heme oxygenase-1 gene via the stress response element pathway. Curr Pharm Des 2003;9:2499–511.
[44] Tanaka Y, Maher JM, Chen C, Klaassen CD. Hepatic ischemia–reperfusion induces renal heme oxygenase-1 via NF-E2-related factor 2 in rats and mice. Mol Pharmacol 2007;71:817–25.
[45] Katsargyris A, Klonaris C, Alexandrou A, Giakoustidis AE, Vasileiou I, Theocharis S. Toll-like receptors in liver ischemia reperfusion injury: a novel target for therapeutic modulation? Expert Opin Ther Targets 2009;13:427–42.
[46] Kleinert H, Schwarz PM, Forstermann U. Regulation of the expression of inducible nitric oxide synthase. Biol Chem 2003;384:1343–64.
[47] Poligone B, Baldwin AS. Positive and negative regulation of NF-kappaB by COX-2: roles of different prostaglandins. J Biol Chem 2001;276:38658–64.
[48] Jin W, Wang H, Yan W, Xu L, Wang X, Zhao X, et al. Disruption of Nrf2 enhances upregulation of nuclear factor-kappaB activity, proinflammatory cytokines, and intercellular adhesion molecule-1 in the brain after traumatic brain injury. Mediators Inflamm 2008;2008:725174.
[49] Ho FM, Kang HC, Lee ST, Chao Y, Chen YC, Huang LJ, et al. The anti-inflammatory actions of LCY-2-CHO, a carbazole analogue, in vascular smooth muscle cells. Biochem Pharmacol 2007;74:298–308.