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Protective effect of heme oxygenase-1 induction against hepatic injury in alcoholic steatotic liver exposed to cold ischemia/reperfusion

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ABSTRACT

Aims: The purpose of this study was to investigate the cytoprotective role of heme oxygenase-1 (HO-1) induction in hepatic injury in alcoholic steatotic liver exposed to cold ischemia/reperfusion (I/R).
Main methods: Animals were fed an ethanol liquid diet or isocaloric control diet for 5 weeks. Isolated perfused rat livers were preserved in Histidine–Tryptophan–Ketoglutarate at 4 °C. After 24 h of storage, livers were subjected to 120 min of reperfusion with Krebs–Henseleit bicarbonate buffer at 37 °C. Animals were pretreated with cobalt protoporphyrin (CoPP, 5 mg/kg, i.p.) or zinc protoporphyrin (ZnPP, 25 mg/kg, i.p.), HO-1 inducer and antagonist, respectively.
Key findings: In the model of ischemia/isolated perfusion, endogenous HO-1 was downregulated in the livers fed with ethanol diet (ED I/R). In ED I/R group, portal pressure and lactate dehydrogenase release were significantly increased, while bile output and hyaluronic acid clearance decreased compared to rats fed on control diet (CD I/R). Furthermore, hepatic glutathione content decreased and lipid peroxidation increased in the ED I/R group compared to the CD I/R group. These alterations were attenuated by upregulation of HO-1 with CoPP pretreatment.
Significance: Our results suggest that chronic ethanol consumption aggravates hepatic injury during cold I/R and it is likely due to downregulation of endogenous HO-1. Prior induction of HO-1 expression may provide a new strategy to protect livers against hepatic I/R injury or to increase the donor transplant pool through modulation of marginal alcoholic steatotic livers.

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Introduction

Liver transplantation is the treatment of choice for patients with end-stage liver disease. However, due to organ shortage, many patients die every year while on the waiting list. Liver transplantation has received renewed interest with the potential use of marginal livers to expand the number of available donors. Alcohol intake and hypernutrition associated with obesity are the major causes of steatotic liver, and liver steatosis is relatively common even in asymptomatic patients. Steatotic livers are frequently discarded by transplant teams due to the risks of primary nonfunction, as compared to normal livers, resulting in further critical shortage of human donor livers (Gao et al., 2009). Insight into the mechanisms of liver injury following ischemia/reperfusion (I/R) of alcoholic steatotic livers could help to develop therapeutic trials to attenuate this injury, thereby permitting the use of these organs for liver transplantation.

A number of studies have suggested that the production of reactive oxygen species (ROS) during I/R of the liver is a major pathophysiological constituent of acute liver failure in an I/R situation. Overproduction of ROS during I/R injury may result in direct cellular damage and subsequent release of proinflammatory mediators (Hirsch et al., 2006). Chronic ethanol treatment also increases ROS production and enhances peroxidation of lipids, protein and DNA in a variety of systems (Cederbaum, 2001). Endogenous antioxidants, such as superoxide dismutase, catalase, and glutathione (GSH) limit the effects of ROS but can become overwhelmed by the large amounts of ROS (He et al., 2006). However, we reported that ascorbic acid acts not only as an antioxidant but also as a prooxidant during cold I/R (Park and Lee, 2008).

Heme oxygenase-1 (HO-1), an inducible heat shock protein 32, is highly induced during oxidative stress response including I/R situation. Since HO-1 is induced as a protective mechanism in response to various stimuli, targeted induction of this cytoprotective enzyme may be considered as an important therapeutic strategy for the protection against inflammatory process and oxidative tissue damage (Katori et al., 2002). HO-1 overexpression maintains tissue architecture and preserves organ function in a cold I/R model (Kato et al., 2001). Furthermore, upregulated HO-1 ameliorates fat Zucker rat livers from cold I/R injury (Amersi et al., 1999). When mice were treated with cobalt protoporphyrin (CoPP) to induce HO-1 expression, ethanol-induced sensitivity to lipopolysaccharide was ameliorated (Mandal et al., 2010). However, recent studies indicate that the protection might be restricted to a narrow threshold of HO-1

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overexpression. Indeed, several studies have shown that excessive overexpression of HO-1 is directly related to increased injury (Suttner and Dennery, 1999; Suttner et al., 1999).

We hypothesized that HO-1 may exert a protective role in alcoholic steatotic livers during I/R insult. Accordingly, we used a model of isolated perfused rat liver (IPRL) to investigate hepatic dysfunction in alcoholic steatotic liver exposed to cold I/R, and the effect of HO-1 on functional recovery of the liver itself excluded from the systemic events by using a HO-1 inducer or inhibitor.

Materials and methods

Animals and induction of alcoholic fatty liver

Male Sprague–Dawley rats weighing 150–170 g were acclimatized to laboratory conditions at Sungkyunkwan University for at least 1 week. Rats were kept in a temperature- and humidity-controlled room (25 ± 1 °C and 55 ± 5%, respectively) with a 12 h light–dark cycle. This study was approved by the Animal Care Committee of Sungkyunkwan University, Korea, and all animals were handled according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health guidelines. Due to the tendency of animals to reduce their solid food consumption when given alcohol, they were fed with a liquid diet. Chronic ethanol feeding was achieved by incorporation of ethanol in a nutritionally adequate liquid diet obtained from Dyets Inc. (Bethlehem, PA, USA). The liquid diet provided 1 kcal/ml, of which 35% of total calories were derived from fat, 47% from carbohydrates, and 18% from protein. Ethanol-treated animals were given diet, where maltose dextrin was isocalorically replaced by ethanol. The alcohol provided 36% of the calories (Lieber and DeCarli, 1989). Diet was fed at a body weight of 150–170 g and ethanol was introduced progressively with 30 g/l of the liquid diet for 2 days, 40 g/l for the subsequent 2 days, followed by the final formula containing 50 g/l (Lieber and DeCarli, 1989; Kerai et al., 1998).

Isolated perfused rat liver (IPRL) and hepatic cold I/R

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg) and systemically heparinized (400 U/kg) via the penile vein. A midline incision was made to the abdomen, and portal vein was cannulated with sterile PE-190 polyethylene tubing and flushed with Krebs–Henseleit bicarbonate buffer (KHBB) containing (in mM) NaCl, 118; KCl, 4.6; $CaCl_2$ 2.5; KH_2PO_4, 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 25; EDTA, 0.1; glucose, 5; sodium taurocholate, 0.03; (pH 7.4, 37 °C) in a non-recirculating system as previously described (Park and Lee, 2008). The flow rate was 4 min/g liver essentially and the perfusate was saturated with a 96% O_2–5% CO_2 gas mixture. After flushing had begun, the inferior vena cava was ligated above the right renal vein and cut distally. During flushing, the liver was dissected free from the rat and moved to the perfusion apparatus. After 10 min of warm perfusion to isolate the liver, cold Histidine–Tryptophan–Ketoglutarate (HTK) solution (Methapharm Inc., Coral Springs, FL, USA) was infused and the liver was immersed in about 40 ml of cold HTK solution inside storage containers. The containers were then placed on ice and stored at 4 °C for 24 h. After cold storage, the liver was reperfused with oxygenated KHBB (pH 7.4, 37 °C) for 120 min. The flow rate was 4 ml/min/g liver essentially during reperfusion.

Experimental groups

To study the effect of HO-1, CoPP, an HO-1 inducer (5 mg/kg) or zinc protoporphyrin (ZnPP), an HO-1 antagonist (25 mg/kg), was administered intraperitoneally to rats fed with control and ethanol diet (CD and ED) 24 h before liver perfusion. Metalloporphyrins (CoPP and ZnPP) were dissolved in 1 ml of 0.2 M NaOH, subsequently adjusted to a pH 7.4 with 1 M HCl, and diluted to the final volume with 0.9% NaCl (Kaizu et al., 2003). The dose and timing of CoPP and ZnPP injection selected were based on previous studies (Ito et al., 2002). The experimental groups were divided into 6 subgroups in the study: (a) IPRL feeding control diet (CD control), (b) IPRL feeding ethanol diet (ED control), (c) cold I/R group feeding control diet (CD I/R), (d) cold I/R group feeding ethanol diet (ED I/R), (e) CoPP treated cold I/R group feeding ethanol diet (CoPP ED I/R), and (f) ZnPP treated cold I/R group feeding ethanol diet (ZnPP ED I/R).

Liver weight to body weight ratio and hepatic triglyceride concentration

The weight of rat whole liver was measured 5 h after reperfusion, and was expressed as liver weight to 100 g body weight ratio. To analyze hepatic triglyceride (TG) content the liver was homogenized in buffer containing 0.25 M sucrose, 50 mM Tris–HCl, and 1 mM EDTA (pH 7.4). Lipids were extracted from the liver homogenate by chloroform:methanol (2:1) and measured according to Folch et al. (1957). Hepatic triglyceride concentration was determined by spectrophotometric procedures using commercial kit (IVDLab Co., Ltd., Uiwang, Korea) with triglyceride (200 mg/dl) as a standard.

Bile output, portal pressure, lactate dehydrogenase (LDH) and hyaluronic acid (HA) clearance

Bile was collected via a cannula (PE-10) placed in the common bile duct. Bile output was measured gravimetrically in prepared tubes. Portal pressure was monitored manometrically from tubing attached to the inflow (Charrueau et al., 2002). As an index of parenchymal cell injury, LDH activity in the perfusate was determined by standard spectrophotometric procedures by ChemiLab LDH assay kit (IVDLab Co.). Perfusate HA clearance was quantified using a commercial rat HA ELISA assay kit (BD biosciences Co., San Diego, CA, USA).

HO-1 activity assay

HO-1 enzyme activity was measured according to a previously described method (Raju and Maines, 1996). Microsomal fraction (200 μl) was added to the reaction mixture (200 μl) containing 0.8 mM NADPH, 2 mM glucose 6-phosphate, 0.2 U glucose-6-phosphate dehydrogenase, 20 μM hemin, 100 mM potassium phosphate buffer, pH 7.4, and 2 mg of rat liver cytosol as a source of biliverdin reductase. Mixtures were incubated at 37 °C for 1 h in the dark, and the samples were left in an ice bath to terminate the reaction. Bilirubin formed was determined by calculating the differences in absorbances between 464 and 530 nm.

Western blot immunoassay

Freshly isolated liver tissue was homogenized in PRO-PREP™ Protein Extraction Solution (Intron Biotechnology Inc., Seongnam, Korea) for whole liver protein extraction. Nuclear protein was isolated from fresh rat livers using NE-PER Nuclear and Cytoplasmic Extraction Reagents, according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA). The liver homogenates for HO-1 (20 μg per well) and nuclear fractions for Nrf2 (20 μg per well) separated 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Bands were immunologically detected using polyclonal antibodies against rat HO-1 (1:1000 dilution, Assay Designs Inc., Ann Arbor, MI, USA), and rat Nrf2 (1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Binding of all antibodies was detected using an ECL detection system (Intron Biotechnology Co., Ltd.), according to the manufacturer's instructions. The intensity of the immunoreactive bands was determined using a densitometric analysis program (Image Gauge V3.12, FUJI PHOTO FILM Co., Ltd, Japan). Density values of HO-1 were

normalized to β-actin (1:2500 dilution, Sigma Chemical Co., St. Louis, MO, USA), and values of nuclear Nrf2 were normalized to Lamin B1 (1:2000 dilution, Abcam, Cambridge, MA, USA).

Total RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Isolation of total RNA was carried out according to the method described by Chomczynski and Sacchi (2006). Reverse transcription of total RNA was performed to synthesize the first strand cDNA using oligo(dT) 12–18 primer and SuperScript™ II RNase H-Reverse Transcriptase (Invitrogen Tech-Line™, Carlsbad, CA, USA). PCR reaction was performed using a diluted cDNA sample and amplified in a 2 μl reaction volume. The final reaction concentrations were as follows: primers, 10 μM; dNTP mixture, 250 mM; 10× PCR buffer; Ex Taq® DNA polymerase, 0.5 U/reaction. For amplification of *HO-1* cDNA, the sequences of primers were 5′-AAG GAG TTT CAC ATC CTT GCA-3′ (sense) and 5′-ATG TTG AGC AGG AAG GCG GTC-3′ (antisense). The primers used for amplification of *β-actin* cDNA as an internal standard were 5′-TTG TAA CCA ACT GGG ACG ATA TGG-3′ (sense) and 5′-GAT CTT GAT CCT TCA TGG TGC TAG-3′ (antisense). PCR products of *HO-1* and *β-actin* were 568 and 764 base pairs (bp) in length, respectively. All PCR reactions had an initial denaturation step at 94 °C for 5 min and a final extension at 72 °C for 7 min using the GeneAmp 2700 thermocycler (Applied Biosystems, Foster City, CA, USA). The PCR amplification cycling conditions applied are as follows: 30 cycles of 45 s at 94 °C, 45 s at 65 °C and 60 s at 73 °C for *HO-1*; 25 cycles of 30 s at 94 °C, 30 s at 62 °C and 60 s at 72 °C for *β-actin*, respectively. After RT-PCR, 10 μl samples of the amplified products were resolved by electrophoresis in 1.5% agarose gel and stained with ethidium bromide. The intensity of each PCR product was evaluated semiquantitatively using a digital camera (DC120, Eastman Kodak, New Haven, CT, USA) and a densitometric scanning analysis program (1D Main, Advanced American Biotechnology, Fullerton, CA, USA).

Hepatic lipid peroxidation and GSH content

The steady-state level of malondialdehyde (MDA), which is the end-product of lipid peroxidation, was analyzed in the liver homogenates by measuring the level of thiobarbituric acid-reactive substances spectrophotometrically at 535 nm as described by Buege and Aust (1978) using 1,1,3,3-tetraethoxypropane (Sigma Chemical Co.) as the standard. Liver tissue samples were determined after precipitation with 1% picric acid, total GSH level was determined in the liver homogenates using yeast-glutathione reductase, 5,5′-dithiobis(2-nitrobenzoic acid), and NADPH, at 412 nm. Oxidized glutathione (GSSG) level was measured by the same method in the presence of 2-vinylpyridine, and the reduced GSH level was calculated as the difference between total GSH and GSSG levels.

Table 1
Liver weight to body weight ratio, starting and final body weights, and hepatic triglyceride content of rats after ethanol diet administration for 5 weeks.

	Liver weight/body weight ratio (g liver/100 g body weight)	Starting body weight (g)	Final body weight (g)	Hepatic triglyceride (mg/g liver)
Control diet	2.81 ± 0.11	175.4 ± 2.1	306.3 ± 2.8	6.15 ± 0.32
Ethanol diet	3.22 ± 0.21**	176.8 ± 2.0	304.7 ± 6.1	10.91 ± 1.07**

Values are means ± S.E.M. for 8–10 rats per group. **Significantly different ($P<0.01$) from control diet group.

Statistical analysis

All results are presented as mean ± S.E.M. The overall significance of the experimental results was examined by two-way analysis of variance. The differences between the groups were considered significant at $P<0.05$, with the appropriate Bonferroni correction made for multiple comparisons.

Results

Effect of chronic alcohol consumption on liver weight and hepatic TG content

At the end of the 5-week diet period, no significant difference was found in the body weights of the experimental groups (data not shown). Liver weight to body weight ratio was 2.81 ± 0.10 g liver/100 g body weight in the animals fed on CD. Chronic alcohol consumption significantly increased liver weight to 3.22 ± 0.21 g liver/100 g body weight. Hepatic TG content in the animals fed on CD for 5 weeks was 6.15 ± 0.32 mg/g liver. In animals fed on ED, hepatic TG content was significantly increased compared to animals fed on CD (Table 1).

Effect of chronic alcohol consumption on portal pressure and HA clearance

As shown in Fig. 1A, portal pressure in each I/R groups was significantly higher compared to each control group. In the ED group, portal

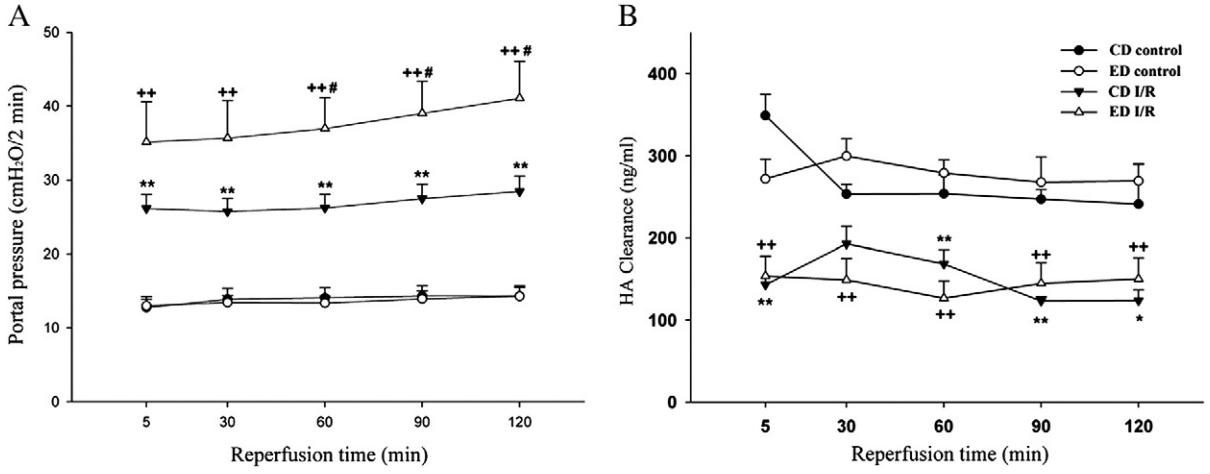

Fig. 1. Effect of chronic alcohol consumption on portal pressure (A) and HA clearance (B) during reperfusion after hepatic cold ischemia. Values are means ± S.E.M. for 8–10 rats per group. *, **Significantly different ($P<0.05$, $P<0.01$) from CD control. ++Significantly different ($P<0.01$) from ED control. #Significantly different ($P<0.05$) from CD I/R.

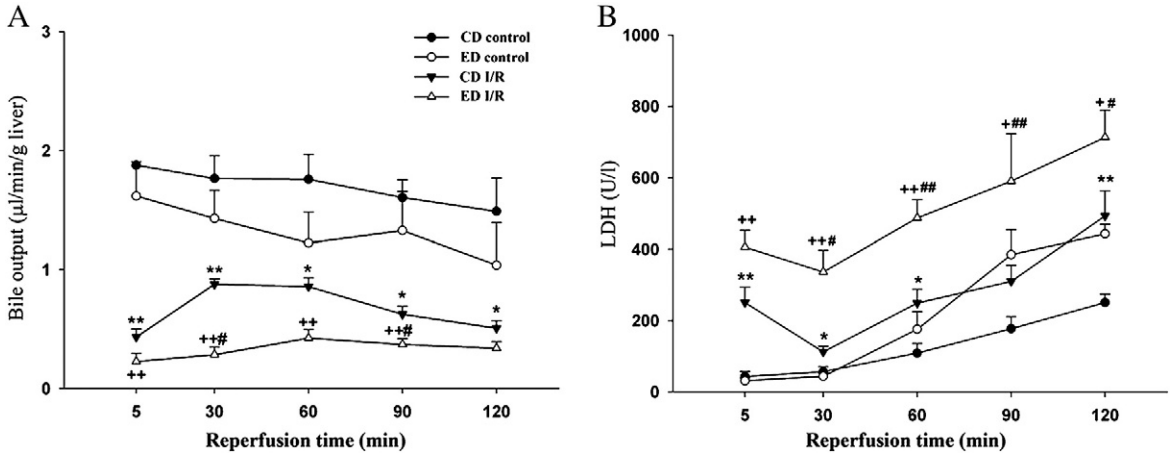

Fig. 2. Effect of chronic alcohol consumption on bile output (A) and LDH release (B) during reperfusion after hepatic cold ischemia. Values are means ± S.E.M. for 8–10 rats per group. *, **Significantly different ($P<0.05$, $P<0.01$) from CD control. +, ++Significantly different ($P<0.05$, $P<0.01$) from ED control. #, ##Significantly different ($P<0.05$, $P<0.01$) from CD I/R.

pressure increased more than that in the CD group after reperfusion. HA clearance in CD and ED I/R group was markedly lowered than that in each control group throughout the 120 min perfusion period, but there was no significant difference between each I/R groups (Fig. 1B).

Effect of chronic alcohol consumption on bile output and LDH release

The bile output in each I/R group was significantly lower than that in the control groups. The decreased level of bile output was more significant in animals fed on ED, especially after 30 and 90 min of reperfusion (Fig. 2A). The LDH level in each I/R group was markedly increased compared to that in each control group. In animals fed on ED, increased level of LDH was more significant than that in the CD group after 30, 60, 90 and 120 min of reperfusion (Fig. 2B).

Effect of chronic alcohol consumption on HO-1 activity, protein, mRNA expression, and nuclear Nrf2 protein expression

In the CD group, HO-1 activity in the cold ischemic group was increased compared to the control group after reperfusion (Table 2). This increase was augmented by CoPP treatment, but was suppressed by ZnPP treatment. In the ED control group, no significant difference in HO-1 activity between the control and I/R group after reperfusion. However, in animals fed on ED, HO-1 activity after 120 min of reperfusion increased significantly by CoPP treatment, but suppressed by ZnPP treatment. The level of HO-1 protein expression increased after reperfusion in the animals fed on CD. This increase was suppressed by ZnPP treatment. In animals fed on ED, there were no significant differences in the level of HO-1 protein expression between the control and I/R groups, and the level of HO-1 protein expression in the ED I/R group was lower than that in the CD I/R group. In animals fed on ED, the level of HO-1 protein expression after reperfusion increased significantly by CoPP treatment. In the CD group, the level of *HO-1* mRNA expression increased significantly compared to that of the control group. In animals fed on ED, there were no significant differences in the level of *HO-1* mRNA expression between the control and the I/R group after reperfusion, and the level of *HO-1* mRNA expression in the ED I/R group was lower than that in the CD I/R group. In animals fed on ED, the level of *HO-1* mRNA expression after 120 min of reperfusion increased significantly by both CoPP and ZnPP treatments (Fig. 3). In the CD group, the Nrf2 translocation into the nucleus significantly increased after I/R. In animals fed on ED, there were no significant differences in the nuclear Nrf2 protein expression level between the control and the I/R group. After I/R, CoPP and ZnPP pretreatment increased Nrf2 translocation into the nucleus in the ED groups (Fig. 4).

Table 2
HO-1 activity after hepatic cold ischemia/reperfusion in alcoholic fatty liver.

Groups		HO-1 activity (pmol/min/mg protein)
Control diet	Control	58.3 ± 2.7
	I/R	78.4 ± 2.7**
	CoPP + I/R	92.4 ± 2.1**,##
	ZnPP + I/R	50.6 ± 4.3#
Ethanol diet	Control	58.0 ± 11.1
	I/R	62.2 ± 6.3#
	CoPP + I/R	88.9 ± 8.0+,$
	ZnPP + I/R	38.3 ± 4.8$

Values are means ± S.E.M. for 8–10 rats per group. **Significantly different ($P<0.01$) from CD control group. #, ##Significantly different ($P<0.05$, $P<0.01$) from CD I/R group. +Significantly different ($P<0.05$) from ED control group. $Significantly different ($P<0.05$) from ED I/R group. HO-1, heme oxygenase-1; I/R, ischemia/reperfusion; CoPP, cobalt protophophyrin; ZnPP, zinc-protophophyrin.

Effect of heme oxygenase-1 on portal pressure and HA clearance in alcoholic liver exposed to cold I/R

During reperfusion, portal pressure in the ED I/R group was significantly higher than that in the control group. Treatment with CoPP dramatically lowered the portal pressure throughout the reperfusion period in ED group, while the ZnPP ED I/R group showed no difference from vehicle-treated ED-ischemic group (Fig. 5A). In animals fed on ED, HA clearance in the I/R group was markedly lowered than that in the control group throughout the 120 min perfusion period. This decrease was suppressed by treatment with CoPP at 5, 30, 60 and 90 min. However, there was no difference in animals pretreated by ZnPP compared to vehicle-treated animals (Fig. 5B).

Effect of heme oxygenase-1 on bile output and LDH release in alcoholic liver exposed to cold I/R

The measured bile output was constant throughout the 120 min period of perfusion. Bile output in the ED I/R group was significantly lower than that in the ED control group. In animals fed on ED, treatment with CoPP significantly increased bile output at 5, 30, 60 and 90 min after reperfusion. However, there was no difference in animals pretreated by ZnPP compared to vehicle-treated animals (Fig. 6A). In animals fed on ED, LDH level in the I/R group markedly increased compared to the control group throughout the reperfusion period. Treatment with CoPP suppressed this increase throughout the

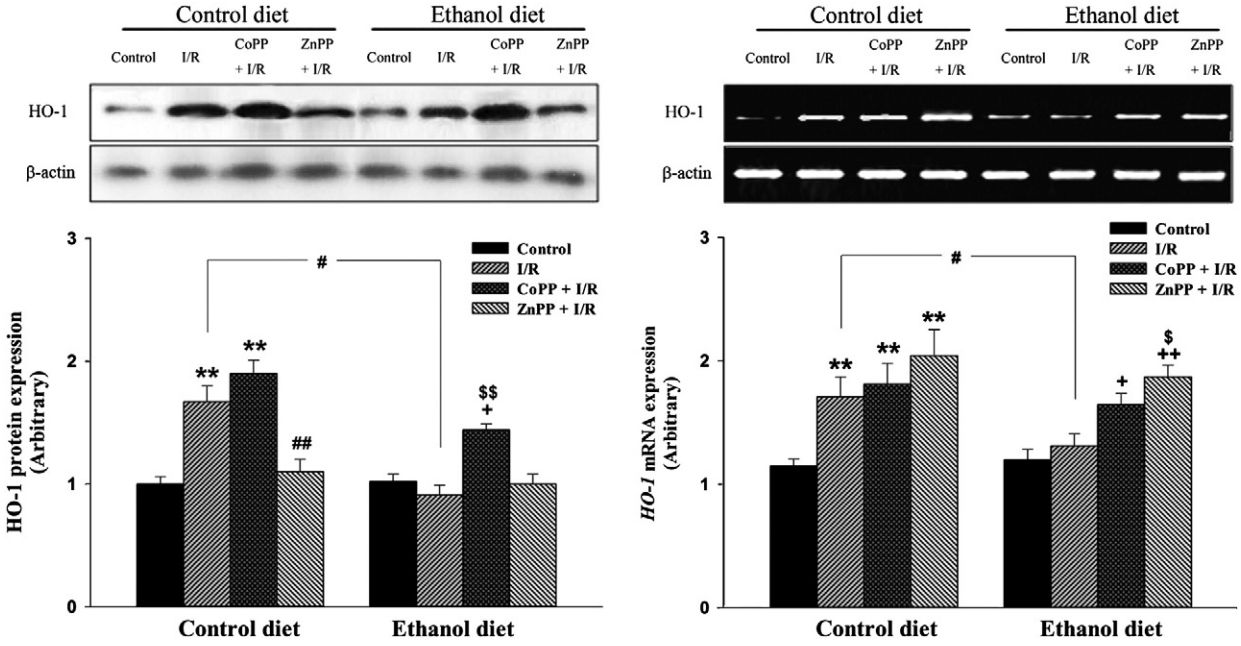

Fig. 3. Effect of chronic alcohol consumption on hepatic HO-1 protein and mRNA expression during reperfusion after hepatic cold ischemia. Values are means ± S.E.M. for 8–10 rats per group. Density values were normalized to β-actin. **Significantly different ($P<0.01$) from CD control. #, ##Significantly different ($P<0.05$, $P<0.01$) from CD I/R. +, ++Significantly different ($P<0.05$, $P<0.01$) from ED control. $, $$Significantly different ($P<0.05$, $P<0.01$) from ED I/R. CoPP, cobalt protophophyrin; ZnPP, zinc protophophyrin.

reperfusion period, while ZnPP-treatment made no difference compared to the ED I/R group (Fig. 6B).

Effect of heme oxygenase-1 in hepatic lipid peroxidation and GSH content in alcoholic steatosis

Hepatic MDA level increased after reperfusion in both CD and ED groups. In the animals fed on CD, CoPP treatment suppressed increase in MDA level after reperfusion. The MDA level in the ED I/R group increased significantly compared to the ED control group and it was higher than that in the CD I/R group. CoPP treatment suppressed this increase. However, there was no change in animals pretreated by ZnPP compared to vehicle-treated animals (Fig. 7A). The hepatic GSH content was 10.17 ± 1.05 nmol/g liver in the CD control. In the CD I/R group, hepatic GSH content decreased to 32% of the CD control group but CoPP treatment suppressed this decrease. In animals fed on ED, hepatic GSH content decreased to 42% of the ED control group after 120 min of reperfusion, and it was lower than that in the CD I/R group. However, CoPP treatment suppressed this decrease, while ZnPP treatment showed no difference from vehicle-treated ischemic group (Fig. 7B).

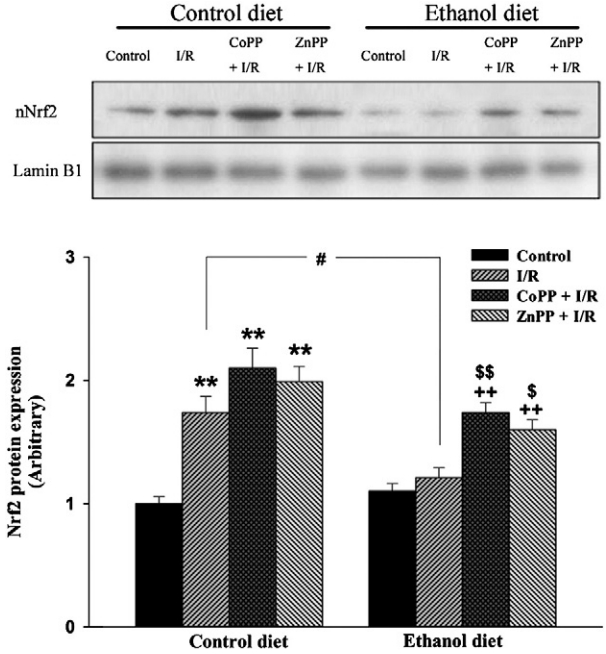

Fig. 4. Effect of chronic alcohol consumption on nuclear Nrf2 protein expression during reperfusion after hepatic cold ischemia. Values are means ± S.E.M. for 8–10 rats per group. Density values of nuclear Nrf2 were normalized to nuclear lamin B1. **Significantly different ($P<0.01$) from CD control. #Significantly different ($P<0.05$) from CD I/R. ++Significantly different ($P<0.01$) from ED control. $, $$Significantly different ($P<0.05$, $P<0.01$) from ED I/R. CoPP, cobalt protophophyrin; ZnPP, zinc protophophyrin.

Discussion

Steatosis is a well-established risk factor for orthotopic liver transplantation and liver resection, and the risk of graft dysfunction and failure are correlated with the severity of fatty infiltration (Urena et al., 1998). Alcoholic fatty liver diseases share a similar spectrum of hepatic histopathologies, such as, steatohepatitis and fibrosis (Yamada et al., 2003). However, studies suggested that chronic alcohol intake can sensitize the liver to a variety of stresses, such as viral hepatitis, hemorrhagic shock, endotoxemic stress and I/R insult (Bauer et al., 1995; Zhong et al., 1995; de Torres and Poynard, 2003; Karaa et al., 2005). We induced alcoholic steatosis using the Lieber—DeCarli liquid diet, which induces mild hepatic injury that mimics the early stages of human alcoholic liver diseases (de la M Hall et al., 2001).

Livers subjected to prolonged preservation are more susceptible to I/R injury and oxidative stress from oxygen free radicals, which have been shown to play a major role in organ injury following reperfusion of ischemic organs (D'Alessandro et al., 1991). HO-1 has been implicated in cytoprotection against oxidative stress in vitro and in vivo. Despite the large number of studies supporting the cytoprotective effect of HO-1, several reports showed contradictory results (Suttner and Dennery, 1999). Although moderate steatosis results in enhanced cold I/R injury (Fukumori et al., 1999), the cellular mechanisms underlying cold I/R injury of alcoholic steatotic livers remain unclear.

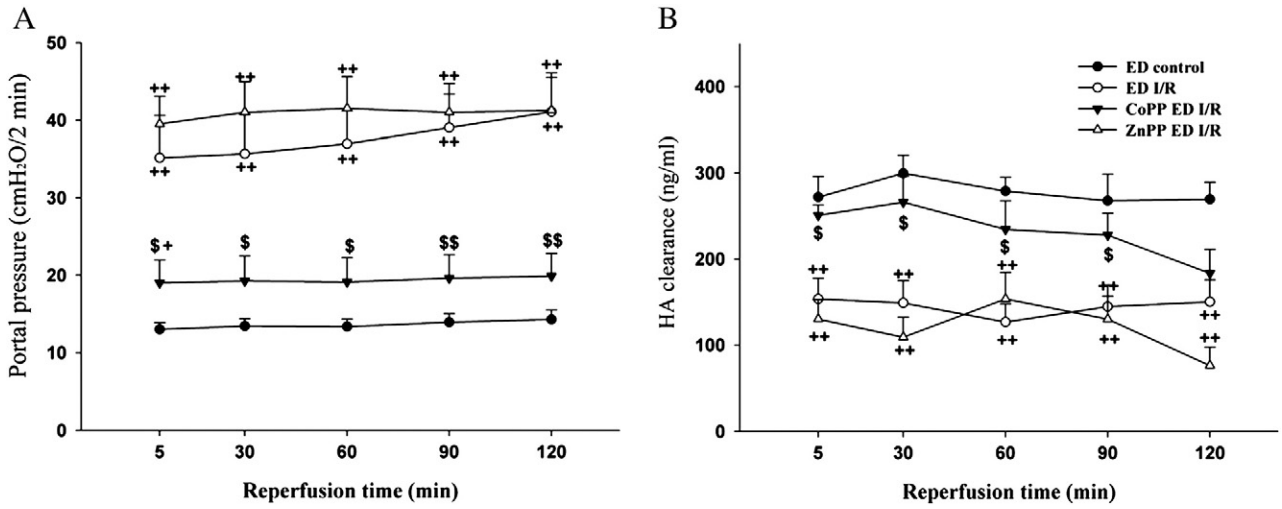

Fig. 5. Effect of HO-1 induction on portal pressure (A) and HA clearance (B) in alcoholic liver exposed to cold I/R. Values are means ± S.E.M. for 8–10 rats per group. +, ++Significantly different ($P<0.05$, $P<0.01$) from ED control. $, $$Significantly different ($P<0.05$, $P<0.01$) from ED I/R. CoPP, cobalt protophophyrin; ZnPP, zinc protophophyrin.

Moreover, the diverse functional sequelae that occur in a multifunctional organ such as the liver remain incompletely understood.

The sinusoidal endothelium is particularly susceptible to ischemic damage, which even under cold storage and free radical injury presumably occurs within the extracellular matrix when the endothelium is altered (Jaeschke and Farhood, 1991). Pathological damage or death of the endothelium may subsequently lead to changes in endothelial and stellate cell contractility, which can result in significant changes in hepatic hemodynamics. Previous studies showed that the severity of steatosis had a greater effect on the microcirculation, and this microcirculatory impairment may result in poor perfusion with cold preservation solution and explain the poor tolerance of steatotic livers to cold preservation (Seifalian et al., 1999). Portal venous blood flow is an indicator of hepatic microcirculation, and a high portal pressure is of clinical relevance as an indicator of hepatic disease severity before transplantation, as well as in patients suffering from acute liver rejection. In this study, the portal pressure was more remarkably elevated in the livers from animals fed on ED than CD animals after reperfusion. HA is rapidly removed from the circulation primarily by the hepatic endothelial cells, which are the specific sites of its degradation (Eriksson et al., 1983; Fraser et al., 1984). As long as increased production from tissues is excluded, impaired uptake and catabolism of HA by hepatic endothelial cell dysfunction result in elevation of serum HA levels. Therefore, HA is a potential indicator of hepatic endothelial cell damage (Deaciuc et al., 1993). In this study, HA clearances were reduced both in the livers from ED-fed animals and CD-fed animals after reperfusion.

Microcirculatory disturbances are responsible for poor hepatocyte function during graft reperfusion (Koeppel et al., 1996). Biliary secretion is a main function of the liver. Bile production is regarded as a reliable and accurate measure of hepatocellular integrity and correlates well with hepatocellular ATP content during both preservation of the donor liver and experimental liver perfusion (Kamiike et al., 1985). When rats were fed on CD and ED, the levels of bile output were decreased during reperfusion after hepatic cold ischemia, and I/R group fed on ED showed more remarkable impairment of bile secretion than that of the I/R group fed on CD throughout reperfusion period. LDH in the perfusate of the isolated perfused liver model is a reliable marker for assessing liver parenchymal cell membrane integrity (Vajdova et al., 2000). After reperfusion, released LDH level was elevated remarkably in the livers from animals fed on ED, which demonstrated clearly that hepatocytes were more severely damaged in the ethanol-fed group, as compared to the control-fed group. Taken together, livers from alcoholic steatotic rats are more susceptible to cold I/R damage, compared to control livers, as reflected by the increased LDH level and portal pressure and decreased bile secretion.

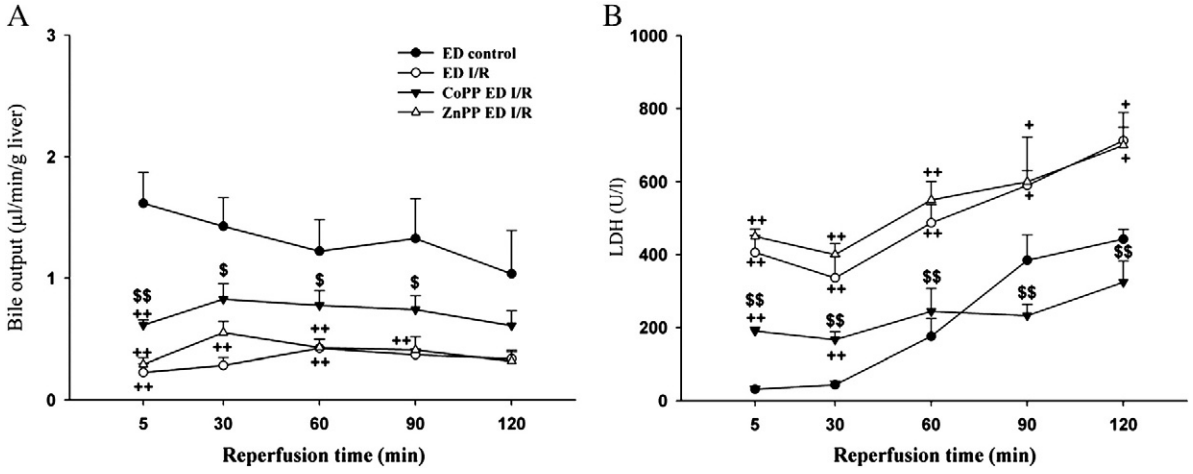

Fig. 6. Effect of HO-1 induction on bile output (A) and LDH release (B) in alcoholic liver exposed to cold I/R. Values are means ± S.E.M. for 8–10 rats per group. +, ++Significantly different ($P<0.05$, $P<0.01$) from ED control. $, $$Significantly different ($P<0.05$, $P<0.01$) from ED I/R. CoPP, cobalt protophophyrin; ZnPP, zinc protophophyrin.

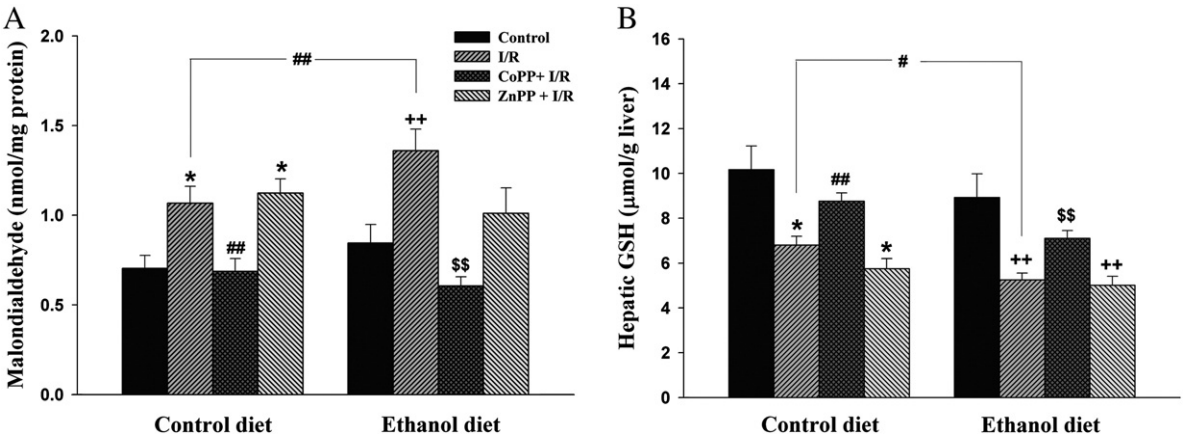

Fig. 7. Effect of HO-1 induction on hepatic MDA (A) and GSH content (B) in alcoholic liver exposed to cold I/R. Values are means ± S.E.M. for 8–10 rats per group. *Significantly different ($P<0.05$) from CD control. #, ##Significantly different ($P<0.05$, $P<0.01$) from CD I/R. ++Significantly different ($P<0.01$) from ED control. $$Significantly different ($P<0.01$) from ED I/R. CoPP, cobalt protophophyrin; ZnPP, zinc protophophyrin.

Previous studies investigated that moderate upregulation of HO-1 expression by HO-1 inducers, hemin or CoPP dramatically suppresses pathological events, and exerts cytoprotective functions in a number of I/R injury transplant models, including heart, liver and kidney (Suttner et al., 1999; Raju and Maines, 1996; Blydt-Hansen et al., 2003; Braudeau et al., 2004). Surprisingly, although potent cytoprotective effects of HO-1 have been documented in a number of I/R injury models, little if any is known about HO-1 upregulation prevents reperfusion injury after cold storage of alcoholic steatotic liver. In this study, HO-1 activity, protein and mRNA expression were markedly increased in the livers of CD animals after reperfusion. In ED-fed animals, HO activity, protein and mRNA expression were not different between control and I/R, suggesting that aggravated damage in the ED group could be due to its lack of HO-1 expression. According to previous studies, the cellular defense mechanism which is considered to be responsible for enhancing HO-1 expression is cytoplasmic redox-sensitive transcription factors such as Nrf2, NF-kB and AP-1. Among these transcription factors, Nrf2 plays a central role in the transcriptional regulation of antioxidant enzymes including HO-1, and is considered one of the major transcription factors for antioxidant response element (ARE) (Camhi et al., 1998; Horikawa et al., 2002). After I/R, translocation of Nrf2 into the nucleus decreased in ED-fed animals compared to CD-fed animals, suggesting that it might be a primary factor of the diminished I/R-induced HO-1 expression in alcoholic steatotic liver. To clarify if HO-1 is associated with the mechanisms of cold I/R injury in alcoholic steatosis, we further conducted experiments with the livers from ethanol-fed animals pre-treated with CoPP and ZnPP. As expected, CoPP augmented HO-1 activity, protein and mRNA expressions after reperfusion in both CD and ED groups. Moreover, HO-1 activity and protein expression levels were decreased in the livers of ZnPP-pretreated animals, while mRNA levels were increased to compensate for reduced activity. The results of our ex vivo cold ischemia model followed by reperfusion show that intensified hepatocellular damage and hepatic malfunction by chronic alcohol consumption were attenuated by overexpression of HO-1, as reflected in less resistance, increased bile output and decreased hepatocyte injury. These results support the hypothesis that HO-1 may play an essential role in the pathogenesis of cold I/R injury of alcoholic steatotic livers.

During I/R, free radicals lead to molecular destruction, including lipid peroxidation, which causes changes in biomembrane-associated functions and structure, resulting in disruption of cells and subcellular organelle physiology. It is well known that the crucial characteristics of HO-1 are redox regulation and its role in the hepatic response to oxidative stress (Bauer and Bauer, 2002). Furthermore, chronic ethanol consumption promotes oxidative stress, by increased formation of ROS and by depletion of oxidative defenses in the cell (Hoek and Pastorino, 2002). HO-1-related antioxidant pathway is a strong candidate of ethanol-induced injury (Yao et al., 2009). GSH is the most important, ubiquitous thiol compound, and works synergistically with other cellular antioxidants to neutralize and scavenge oxygen as well as other free radical species, thereby preventing or diminishing oxidative stress. Decreased hepatic GSH levels increase susceptibility to anoxic damage and oxidative stress in hepatocytes, while an optimal level of GSH reduces hepatic necrosis and improved liver function following cold storage (Bilzer et al., 1999). In cold ischemic livers, hepatic GSH content was lowered after reperfusion in both CD and ED groups, and this alteration was more remarkable in ethanol-fed animals. In contrast, the level of hepatic lipid peroxidation increased after reperfusion in both CD and ED groups, and this was more prominent in the livers from animals fed on ED. CoPP-induced HO-1 overexpression attenuated a reduction in hepatic GSH content and lipid peroxidation, consistent with previous reports that demonstrated the antioxidative properties of HO-1 (Gonzales et al., 2006; Kruger et al., 2006). Our results suggest that alcoholic steatotic livers are more susceptible for oxidative damages against cold I/R via decreased expression by changing the intracellular redox state.

Conclusions

Severe parenchymal cell injury and deterioration of hepatic microcirculation were observed during cold ischemia, and followed reperfusion in alcoholic steatotic liver. It is may be due to downregulation of endogenous HO-1 induction. Pharmacological HO-1 upregulation protects severe cold I/R injury in alcoholic steatotic liver. Furthermore, these results suggest that oxidative stress is a cause of deterioration in the hepatic microcirculation and subsequent hepatocellular malfunction, which were also prevented by HO-1 upregulation. Therefore, prior induction of HO-1 expression may provide a therapeutic new strategy to protect livers against hepatic I/R injury, thereby increasing the donor transplant pool through modulation of marginal alcoholic steatotic livers.

Conflict of interest

The authors declare that there are no conflicts of interest.

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