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Role of Kupffer Cells in the Vasoregulatory Gene Expression during Hepatic Ischemia/Reperfusion

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Hepatic microcirculatory failure is a major component of reperfusion injury in the liver. Recent data provided some evidence that endothelium-derived vasoconstrictors and vasodilators may be functionally important to the control of the total hepatic blood flow under these conditions of circulatory failure. Since Kupffer cells provide signals that regulate the hepatic response in ischemia/reperfusion (I/R), the aim of this study was to investigate the role of Kupffer cells in the I/R-induced imbalance of vasoregulatory gene expression. Rats were subjected to 60 min hepatic ischemia, followed by 5 h of reperfusion. The Kupffer cells were inactivated by gadolinium chloride ($GdCl_3$, 7.5 mg/kg body weight, intravenously) 1 day prior to ischemia. Liver samples were obtained 5 hrs after reperfusion for RT-PCR analysis of the mRNA for genes of interest: endothelin-1 (ET-1), its receptors ET_A and ET_B, endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) and heme oxygenase-1 (HO-1). ET-1 mRNA expression was increased by I/R. mRNA levels for ET_A receptors showed no change, whereas ET_B receptor transcripts increased in the I/R group. The increases in ET-1 and ET_B mRNA were not prevented by the $GdCl_3$ pretreatment. The mRNA levels for iNOS and eNOS significantly increased within the I/R group with no significant difference between the I/R group and the $GdCl_3$-treated I/R group. HO-1 mRNA expression significantly increased in the I/R group and this increase was attenuated by $GdCl_3$. In conclusion, we have demonstrated that an imbalance in hepatic vasoregulatory gene expression occurs during I/R. Our findings suggest that the activation of Kupffer cells is not required for I/R-induced hepatic microvascular dysfunction.

Key words: Hepatic ischemia/reperfusion, Microcirculation, Vasoregulatory gene, Kupffer cells, Gadolinium chloride

INTRODUCTION

Microcirculatory failure during reperfusion is an important factor leading to tissue damage after ischemia. There is substantial evidence to suggest that the integrity of microcirculation during reperfusion is a critical determinant for the survival of various tissues after ischemia (Nevalainen *et al.*, 1986). However, the mechanisms responsible for microvascular failure during reperfusion remain unclear.

Liver microcirculation is normally maintained under the fine balance of vasoconstrictors and vasodilators, such as endothelin-1 (ET-1), nitric oxide (NO) and carbon monoxide (CO) (Pannen *et al.*, 1996). Whereas in the liver, during endotoxemic shock or hepatic I/R, this fine balance is disrupted and results in dysregulation of the hepatic microcirculation (Sonin *et al.*, 1999).

ET-1 is a potent vasoactive peptide that mediates either vasoconstriction or vasodilation depending on its two receptor types, ET_A or ET_B. It was shown that the production of ET-1 is controlled at the transcriptional level and the up-regulation of preproET-1 mRNA can be induced by cytokines, growth factors or hormones (Stephenson *et al.*, 1994). In an intact liver, the vasoconstricting action of ET-1 is finely balanced with the vasodilating action of NO and CO, which are produced by endothelial nitric oxide synthase (eNOS) and heme oxygenase-2 (HO-2). The production of NO and CO increase in liver under certain stress conditions through the stimulation of the inducible enzymes

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iNOS and HO-1 and then they may sufficiently contribute to microcirculatory dysfunction (Fleming *et al.*, 1991). However, the mechanism of regulating the vasoregulatory gene expression during I/R has not yet been investigated.

Kupffer cells, resident macrophages of the liver, are known to produce a wide variety of biologically toxic mediators, such as reactive oxygen species, tumor necrosis factor-α (TNF-α), interleukin-1, and prostaglandin E_2, and these cells have been strongly implicated in the pathogenesis of hepatic injury in various animal models (Shiratori, 1988). It was shown that the inactivation of Kupffer cells with gadolinium chloride inhibits the early injury phase during reperfusion (Jaeschke and Farhood, 1991). However, few studies have examined the direct effect of I/R on Kupffer cell function *in vivo*. Furthermore, no information is available on Kupffer cell-mediated microcirculatory disturbance during I/R.

Therefore, it was hypothesized that activated Kupffer cells are involved in the mechanisms of I/R-induced liver injury. Accordingly, the purpose of this study was to determine if Kupffer cells are indeed involved in hepatic vasoregulatory dysfunction during hepatic I/R.

MATERIALS AND METHODS

Chemicals

Gadolinium chloride ($GdCl_3$), diethylpyrocarbonate (DEPC), ethidium bromide (EtBr), and the alanine aminotransferase (ALT) kit were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Deoxyribonucleotide triphosphate (dNTP), E. coli RNase H, dithiothreitol (DTT), oligo$(dT)_{12-18}$ primer, and SuperScript™ II RNase H⁻ Reverse Transcriptase were supplied from Invitrogen Tech-Line™ (Carlsbad, CA, USA). All other chemicals used were of reagent grades and were locally or commercially available.

Animals

The male Sprague-Dawley rats, weighing 260-320 g, were supplied by the Jeil Animal Breeding Company of Korea and were acclimatized to laboratory conditions at Sungkyunkwan University for at least one week, with food and tap water supplied *ad libitum*.

Pretreatment with $GdCl_3$ and experimental groups

To deplete Kupffer cells *in vivo*, $GdCl_3$ was dissolved in sterilized physiological saline solution, and a dose 7.5 mg/kg body weight was injected 24 h prior to I/R via the tail vein (Hisama *et al.*, 1996). In sham-operated rats, the same volume of saline solution was injected in the same manner as $GdCl_3$. Four experimental groups were studied: (a) sham, (b) $GdCl_3$ (not performed I/R), (c) I/R, and (d) $GdCl_3$-pretreated I/R.

Hepatic ischemia/reperfusion

After overnight fasting, the rats were anesthetized with pentobarbital sodium (40 mg/kg body weight, i.p.). The liver was exposed through a midline incision, and the pedicles of the left and median lobes were occluded with a microvascular clamp for 60 min. This allowed the portal venous flow from the mesenteric circulation to be shunted through the right lobes of the liver, thus, preventing intestinal venous congestion. At the end of the ischemic period, the clamp was removed to allow reperfusion. Sham-operated rats were prepared with the same procedure but without placing the clamp in the left and median lobes. Five hours after the reperfusion, blood was drawn from the abdominal aorta under anesthesia. The livers were excised, immediately frozen in liquid nitrogen, and stored at -70°C until assayed.

Serum alanine aminotransferase activity

ALT activities were determined by spectrophotometric procedures of the Sigma diagnostics INFINITY™ kits 52-UV.

Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was isolated from approximately 100 mg of liver tissue which was obtained from left lobe. In brief, the liver tissue was homogenized with 1 mL of TRIZOL® reagent (Gibco BRL, USA). After the extraction of total RNA with chloroform, the extract was precipitated from the aqueous phase by the addition of isopropanol and was washed with 75% ethanol. Then, it was dissolved in DEPC-treated deionized water and was stored at -75°C. Reverse transcription of total RNA was performed to synthesize the first cDNA strand using oligo$(dT)_{12-18}$ primer and SuperScript II RNase H⁻ Reverse Transcriptase. The reaction of the reverse transcriptase was stopped by incubating it at 70°C for 10 min. The reaction products (cDNAs) were immediately stored at -20°C until PCR analysis. PCR was carried out in each 20 μL volume reaction using gene specific primers (Table I), according to the following protocol: 2 μL of 2.5 mM dNTP, 2 μL of 10X PCR buffer, 10 pmol of each primer for the appropriate target sequence, 14.4 μL of DEPC-treated DW, 0.5 U/reaction Ex *Taq*® DNA polymerase. The PCR was done in a GeneAmp 2700 thermocycler (Perkin-Elmer, Norwalk, USA). PCR amplification cycling (denaturation, annealing, extension) conditions were as follows: 94°C 45 s, 65°C 45 s, 73°C 60 s, 30 cycles for ET-1 and eNOS; 94°C 45 s, 65°C 45 s, 73°C 60 s, 26 cycles for ET_A, ET_B, iNOS and HO-1; 94°C 30 s, 62°C 30 s, 72°C 60 s, 25 cycles for β-actin. All PCR reactions had an initial denaturation step at 95°C for 3 min and a final extension step at 72°C for 7 min. To validate the RT-PCR results, β-actin specific

Table I. PCR primers utilized in the experiment

Gene	Primer sequences (5' → 3')	Product length (bp)
ET-1	sense : TCTTCTCTCTGCTGTTTGTGGCTT anti-sense : TCTTTTACGCCTTTCTGCATGGTA	407
ET_A	sense : AGTGCTAATCTAAGCAGCCAC anti-sense : CAGGAAGCCACTGCTCTGTAC	491
ET_B	sense : AGCTGGTGCCCTTCATACAGAAGGC anti-sense : TGCACACCTTTCCGCAAGCACG	919
iNOS	sense : TTCTTTGCTTCTGTGCTTAATGCG anti-sense : GTTGTTGCTGAACTTCCAATCGT	1061
eNOS	sense : TGGGCAGCATCACCTACGATA anti-sense : GGAACCACTCCTTTTGATCGAGTTAT	202
HO-1	sense : AAGGAGTTTCACATCCTTGCA anti-sense : ATGTTGAGCAGGAAGGCGGTC	568
β-Actin	sense : TTGTAACCAACTGGGACGATATGG anti-sense : GATCTTGATCTTCATGGTGCTAG	764

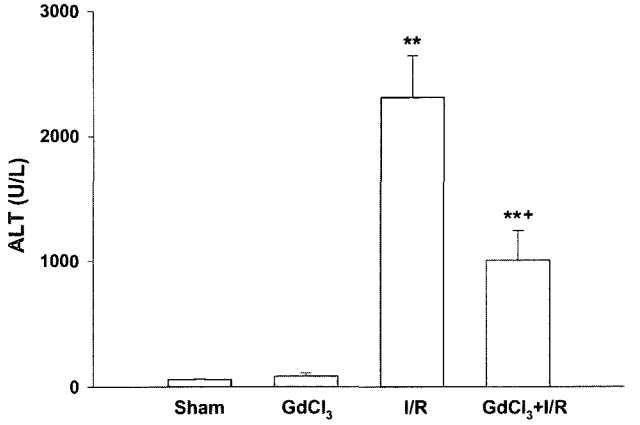

Fig. 1. Effect of $GdCl_3$ on serum ALT activity after ischemia and subsequent reperfusion in rats. Each value is mean±S.E.M. for 7 rats per group. **Significantly different (P<0.01) from sham. *Significantly different (P<0.05) from I/R. $GdCl_3$, gadolinium chloride; I/R, ischemia/reperfusion.

primers were used in the PCR as a housekeeping gene. Reverse transcription of the total RNA was performed to synthesize the first cDNA strand using oligo$(dT)_{12\text{-}18}$ primer and SuperScript II RNase H^- Reverse Transcriptase.

RT-PCR product detection and densitometric analysis

Following RT-PCR, 10 μL aliquot of each amplified product was resolved by gel electrophoresis using a 1.5% agarose gel and stained with EtBr. The intensity of each PCR product was semi-quantitatively evaluated using a digital camera (DC 120, Eastman Kodak, New haven, CT, USA) and a densitometric scanning analysis program (1D Main, Advanced American Biotechnology, CA, USA).

Statistical analysis

All data are presented as means±S.E.M. One-way analysis of the variance (ANOVA) followed by Dunnett's *t*-test was used to determine the statistical significance of the differences between experimental groups. The results were considered significant for a P<0.05.

RESULTS

Serum ALT activity

The serum ALT level in the sham-operated group was 60 ± 4 U/L. Although no apparent changes in the ALT level were seen in $GdCl_3$ alone, the ALT level was markedly elevated to 2313 ± 331 U/L in the I/R group. This increase in the ALT level was significantly suppressed by the $GdCl_3$-treatment (Fig. 1).

Vasoconstrictor genes expression

Changes in the expression of vasoconstrictor genes, ET-1, and its receptor (ET_A and ET_B) during I/R are shown in Fig. 2, 3, and 4. The level of ET-1 mRNA significantly increased in the I/R group when compared with the sham-operated group. There were no significant differences in the ET-1 mRNA expression between the I/R group and the $GdCl_3$-treated I/R group (Fig. 2). Although no apparent changes in ET_A mRNA level were seen in either $GdCl_3$ alone and I/R alone compared to sham, the expression of ET_A mRNA markedly decreased in the $GdCl_3$-treated I/R group (Fig. 3). The level of ET_B receptor mRNA significantly increased in the I/R group compared to the sham-operated group. $GdCl_3$ pretreat-

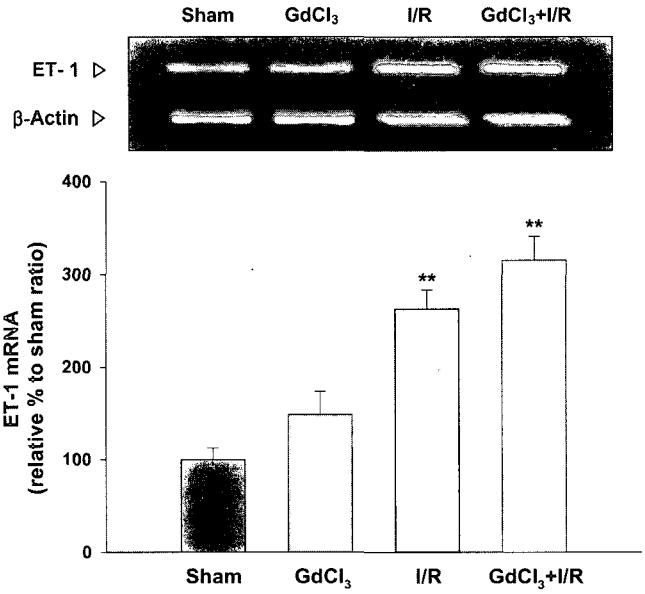

Fig. 2. Effect of $GdCl_3$ on mRNA levels of ET-1 after ischemia and subsequent reperfusion in rats. Each value is mean±S.E.M. for 6-7 rats per group. **Significantly different (P<0.01) from sham.

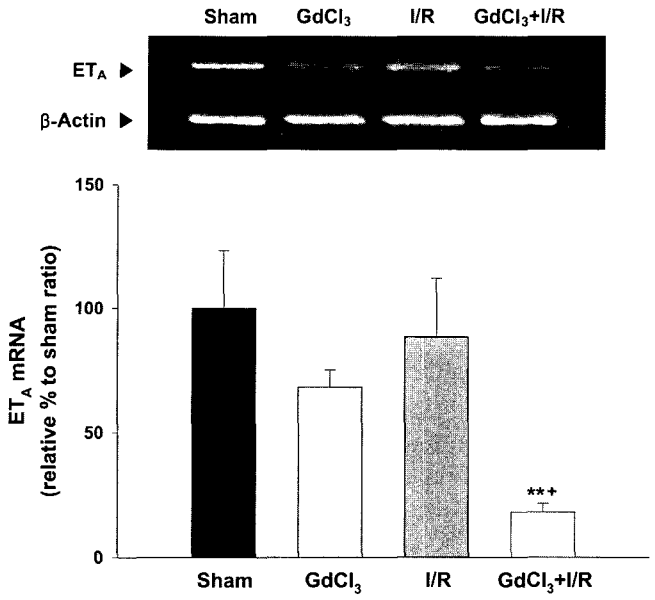

Fig. 3. Effect of $GdCl_3$ on mRNA levels of ET_A receptor after ischemia and subsequent reperfusion in rats. Each value is mean±S.E.M. for 6-7 rats per group. **Significantly different ($P<0.01$) from sham. †Significantly different ($P<0.05$) from I/R.

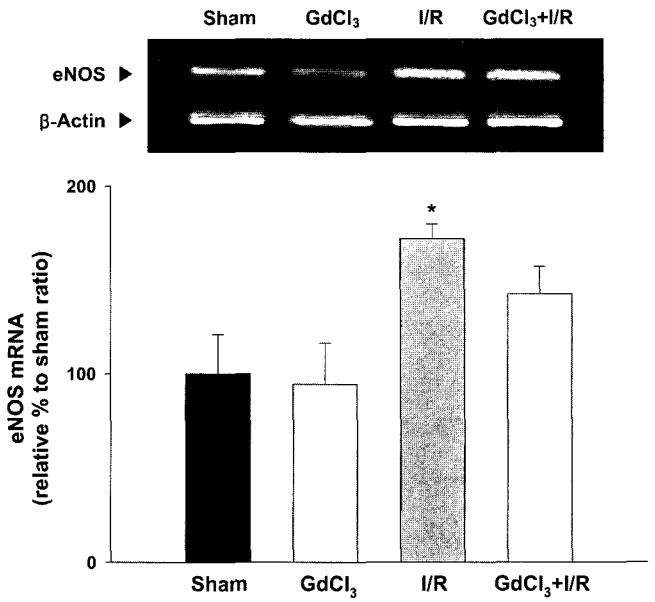

Fig. 5. Effect of $GdCl_3$ on mRNA levels of eNOS after ischemia and subsequent reperfusion in rats. Each value is mean±S.E.M. for 6-7 rats per group. *Significantly different ($P<0.05$) from sham.

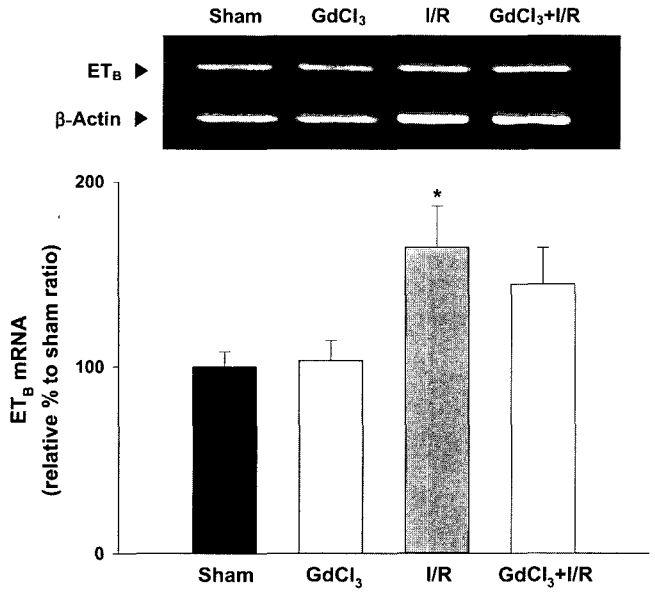

Fig. 4. Effect of $GdCl_3$ on mRNA levels of ET_B receptor after ischemia and subsequent reperfusion in rats. Each value is mean±S.E.M. for 6-7 rats per group. *Significantly different ($P<0.05$) from sham.

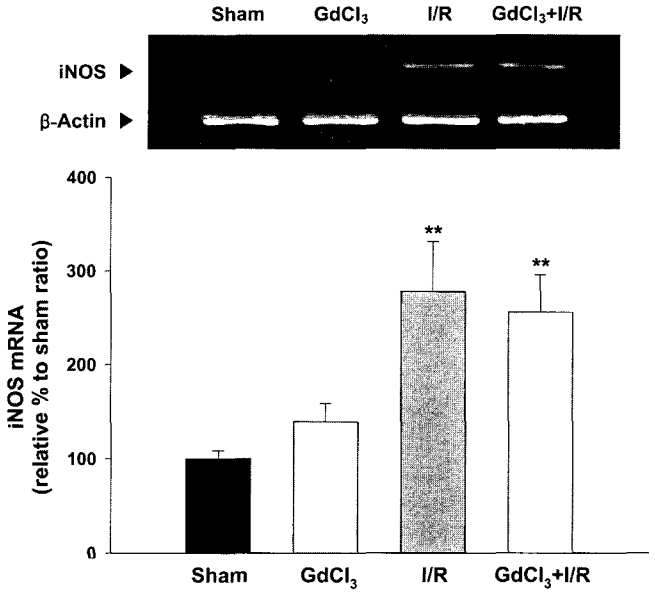

Fig. 6. Effect of $GdCl_3$ on mRNA levels of iNOS after of ischemia and subsequent reperfusion in rats. Each value is mean±S.E.M. for 6-7 rats per group. **Significantly different ($P<0.01$) from sham.

ment did not affect the ET_B receptor mRNA expression during I/R (Fig. 4).

Vasodilator genes expression

As shown in Fig. 5, the level of eNOS mRNA significantly increased in the I/R group compared to the sham-operated group. In the $GdCl_3$-pretreated I/R group, this increase was slightly attenuated but was not significant. There was a very low level of iNOS mRNA in the sham-operated group. However, the level of iNOS mRNA in the I/R group markedly increased; a increase that was not prevented by the $GdCl_3$ pretreatment (Fig. 6). As shown Fig. 7, the level of HO-1 mRNA significantly increased in the I/R group as compared to the sham-operated group.

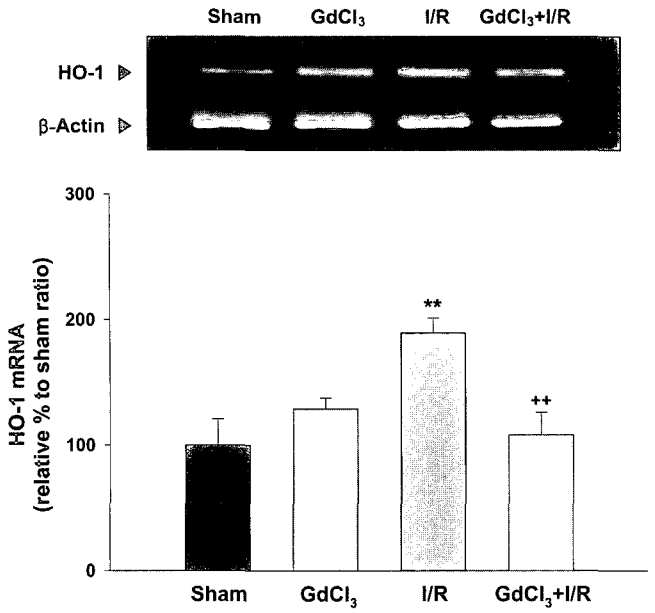

Fig. 7. Effect of $GdCl_3$ on mRNA levels of HO-1 after ischemia and subsequent reperfusion in rats. Each value is mean±S.E.M. for 6-7 rats per group. **Significantly different ($P<0.01$) from sham. ++Significantly different ($P<0.01$) from I/R.

This increase was significantly attenuated by the $GdCl_3$ pretreatment.

DISCUSSION

I/R-induced hepatic injury is one of the major problems after transplantation of the liver (Thurman *et al.*, 1988). An accumulating body of evidence suggests that failure of the hepatic microcirculation is a major component of reperfusion injury in the liver. There is evidence that TNF-α and reactive oxygen species released from neutrophils and/or monocytes are found to be involved in the I/R-induced tissue injury, such as in the heart, lung, and intestine (Kennedy *et al.*, 1989). The pathogenic effect of activated Kupffer cells on hepatic cells has been studied in several recently published reports, and their findings seem to be controversial. It has been shown that cytotoxic products of the Kupffer cells have detrimental effect on hepatocytes and endothelial cells (Arii *et al.*, 1994). Improved survival was demonstrated after the transplantation of Kupffer cell-depleted rat livers (Marzi *et al.*, 1991), which suggested that Kupffer cell elimination has a beneficial effect on postischemic reperfusion damage in liver grafts. On the other hand, Imamura *et al.* (1995) demonstrated that Kupffer cell depletion in the isolated rat liver did not reduce reperfusion injury to sinusoidal endothelial cells, and this was confirmed by transplantation experiments in which no improved survival of rats with rat liver grafts depleted of Kupffer cells was found.

A widely accepted hypothesis proposes that reductions in microvascular perfusion would be the first sign of liver injury and, as a result, predispose the parenchyma to injury. In the present study, I/R markedly increased serum ALT activity, and this increase was significantly attenuated by the $GdCl_3$ pretreatment. This result indicates that Kupffer cells do take part in the liver parenchymal injury during hepatic I/R. Chun *et al.* (1994) has shown that physical prevention of microvascular shut down, using a flow-controlled reperfusion mode, largely prevents parenchymal cell necrosis in an isolated perfused rat liver after I/R. For example, the degree of microcirculatory failure determined the extent of lethal hepatocyte injury. However, the vasoactive mediators involved in the regulation of sinusoidal flow during reperfusion following hepatic ischemia need to be identified. Recent data has provided some evidence that endothelium-derived vasoconstrictors and vasodilators may be functionally important to the control of total hepatic blood flow under I/R. Changes in the gene expression pattern which are triggered by stress factors through the activation of different signal transduction pathways, appear to be an essential part of pathophysiological mechanisms operating in the liver under stress (Wang *et al.*, 1995). Changes in the genes related to vasoregulation may underlie microcirculatory dysfunction that develops in the liver in various pathological conditions, *i.e.* I/R.

In our study, we used a moderate stress model: 60 min of ischemia followed by 5 h of reperfusion. This condition does not cause an acute shut down or a sudden increase in permeability, but it induces changes in the responsiveness of the vasculature that is defected with increased contractility of sinusoids (Pannen *et al.*, 1996) or sinusoid narrowing (Okumura *et al.*, 1994) after infusion of endothelin. Our data show that the level of ET-1 mRNA significantly increased during hepatic I/R. This finding is consistent with previous reports where liver tissue concentrations and hepatic venous plasma levels of ET-1 markedly increased during hepatic I/R (Nakamura *et al.*, 1995). Pretreatment with $GdCl_3$ did not influence the ET-1 mRNA expression. Along with the increase of ET-1 mRNA level, we observed approximately a 2-fold increase in the ET_B mRNA level in the ischemia-reperfused liver, which is in consistent with previous reports of higher ET_B receptor proportion during I/R (Yukoyama *et al.*, 2002). $GdCl_3$ did not influence the expression of ET_B mRNA, and thus, this suggests that Kupffer cells are not essential in the regulation of ET-1 and ET_B receptor gene expression during I/R.

On the other hand, the level of ET_A mRNA transcript slightly decreased 5 h after reperfusion and further decreased in the $GdCl_3$-treated I/R group, suggesting that Kupffer cells upregulate ET_A mRNA expression during I/R. The increase in ET_B receptor expression coupled with no significant change in the expression of ET_A receptors would

predict a predominant effect on presinusoidal constrictor response where the ET_B receptor effects have been reported to be mediated (Zhang *et al.*, 1994). Thus, the increase in the available ET_B receptors would produce an increase in the presinusoidal constrictor response to ET-1, which acts on ET_B as well as ET_A receptors. ET_{B1} receptors expressed on endothelial cells are coupled to the eNOS and thus, mediate vasodilation in the liver while ET_{B2} receptors mediate vasoconstriction (Higuchi and Satoh, 1997). Our results showed that the steady-state mRNA level of eNOS was upregulated during I/R. This suggests that a substantial portion of the constrictor effects of ET-1 may be mediated by ET_{B2} receptors, but this effect is masked by the simultaneous release of NO, which is mediated by ET_{B1} receptors.

To examine the counterbalancing force to the pressor endothelin, we also determined the gene expression of iNOS and HO-1. NO and CO are strong vasodilators and most likely act in concert. Under normal conditions, NO is produced by eNOS in sinusoidal endothelial cells, but inflammatory response induces the iNOS in multiple cell types in the liver including Kupffer cells, hepatocytes and hepatic stellate cells (Clemens *et al.*, 1994). In our study, we found approximately a 3-fold increase in the level of iNOS mRNA in the I/R liver. However, there were no differences in the level of iNOS mRNA between the I/R and $GdCl_3$-treated I/R groups. These results suggest that Kupffer cells might be the minor source of the increased iNOS mRNA expression induced by I/R. HO-1, also known as heat shock protein 32, is induced by oxidative stress, heat shock, and other potentially injurious stimuli that induces NO (Bauer *et al.*, 1998). According to early studies, Kupffer cells act as paracrine regulators of the heme oxygenase-1 gene in hepatocytes after hemorrhagic shock. Furthermore, HO-1 mRNA induction is apparently a general response to various oxidative stresses in mammalian cells (Applegate *et al.*, 1991), and it has been reported that HO induction mediates cytoprotection against experimental oxidative damage *in vitro* and *in vivo* (Abraham *et al.*, 1995). In our experiment, the level of HO-1 mRNA significantly increased in the I/R group. This increase in HO-1 mRNA expression was significantly suppressed by the $GdCl_3$ pretreatment. These results show that Kupffer cells play a major role in the expression of HO-1 mRNA during I/R.

Changes in mRNA expression reported here, if balanced in terms of constrictors-dilators expression, serve to protect against injury and can be considered as a method of preconditioning for future stresses. However, an imbalanced production of vasoactive mediators will result in priming of the vasculature and a greater damage in the occurance of a second stress. Our findings suggest that I/R causes imbalanced hepatic vasoregulatory gene expressions through Kupffer cell-independent mechanisms.

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