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Expression of Hepatic Vascular Stress Genes Following Ischemia/Reperfusion and Subsequent Endotoxemia

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Hepatic ischemia and reperfusion (I/R) predisposes the liver to secondary stresses such as endotoxemia, possibly *via* dysregulation of the hepatic microcirculation secondary to an imbalanced regulation of the vascular stress genes. In this study, the effect of hepatic I/R on the hepatic vasoregulatory gene expression in response to endotoxin was determined. Rats were subjected to 90 min of hepatic ischemia and 6 h of reperfusion. Lipopolysaccharide (LPS, 1 mg/kg) was injected intraperitoneally after reperfusion. Plasma and liver samples were obtained 6 h after reperfusion for serum aminotransferase assays and RT-PCR analysis of the mRNA for the genes of interest: endothelin-1 (ET-1), its receptors ET_A and ET_B, endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), heme oxygenase-1 (HO-1), cyclooxygenase-2 (COX-2), and tumor necrosis factor-α (TNF-α). The activities of serum aminotransferases were significantly increased in the I/R group. This increase was markedly potentiated by LPS treatment. The ET-1 mRNA was increased by LPS alone, and this increase was significantly greater in both the I/R alone and I/R + LPS groups compared to the sham. There were no significant differences in ET_A receptor mRNA levels among any of the experimental groups. ET_B mRNA was increased by both LPS alone and I/R alone, with no significant difference between the I/R alone and I/R + LPS groups. The eNOS and HO-1 transcripts were increased by I/R alone and further increased by I/R + LPS. The iNOS mRNA levels were increased by I/R alone, but increased significantly more by both LPS alone and I/R + LPS compared to I/R alone. The TNF-α mRNA levels showed no change with I/R alone, but were increased by both LPS alone and I/R + LPS. The COX-2 expression was increased significantly by I/R alone and significantly more by I/R + LPS. Taken collectively, significantly greater induction of the vasodilator genes over the constriction forces was observed with I/R + LPS. These results may partly explain the increased susceptibility of ischemic livers to injury as a result of endotoxemia.

Key words: Hepatic ischemia/reperfusion, Endotoxemia, Microcirculation, Vascular stress genes, Lipopolysaccharide

INTRODUCTION

Hepatic ischemia/reperfusion (I/R) is a common problem encountered in many clinical conditions, such as liver transplantation, hepatic failure after shock and liver surgery, trauma and cancer. As a preexisting condition, I/R predisposes the liver to secondary stresses, such as endotoxemia, a common complication of I/R often associated with high mortality (Anderson and Harken, 1990). The mechanism of increased susceptibility to endotoxemia in I/R is not completely understood, although endotoxemia alone has been shown to cause hepatic microcirculatory failure and subsequent liver injury (Ohuchi *et al.*, 1995).

Microcirculatory failure during reperfusion is an important factor leading to tissue damage after ischemia (Nevalainen *et al.*, 1986). Normally, the microcirculation of the liver is maintained by the fine balance of vasoconstrictors and vasodilators, of which endothelin-1 (ET-1) is the primary constrictive force, while nitric oxide (NO) and carbon monoxide (CO), synthesized by nitric oxide synthase (NOS) and heme oxygenase (HO), respectively, comprise the primary dilatory forces in the liver (Pannen *et al.*, 1996). Although the changes in the transcripts of the genes related to the vascular mediators in rat liver

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subjected to I/R (Kim and Lee, 2004) were recently reported, the effect of acute endotoxemia as a secondary stress on the gene expression of vascular mediators in the ischemic liver is unknown.

ET-1 is a potent vasoactive peptide that mediates either vasoconstriction or vasodilation depending on one of its two receptor types, ET_A or ET_B. The ET_A receptor mediates vasoconstriction when it is bound by ET-1. On the other hand, the ET_B type receptor can mediate either vasoconstriction or vasodilation, depending on the ET_B receptor subtype, ET_{B1} or ET_{B2} (Housset *et al.*, 1993). It was shown that production of ET-1 is controlled at the transcriptional level and up-regulation of prepro-ET-1 mRNA can be induced by cytokines, growth factors, or hormones (Stephenson *et al.*, 1994). In the liver, the vasoconstricting action of ET-1 is finely balanced with the vasodilating action of NO and CO, produced by endothelial nitric oxide synthase (eNOS) and HO-2. The production of NO and CO increase in the liver under certain stress conditions through stimulation of the inducible enzymes, iNOS and HO-1, and then may sufficiently contribute to microcirculatory dysfunction (Fleming *et al.*, 1991).

Therefore, the present studies were performed to elucidate the response of the vascular stress genes to the secondary stress of endotoxemia in preexisting hepatic I/R.

MATERIALS AND METHODS

Chemicals

The lipopolysaccharide (LPS, *Escherichia coli*), diethylpyrocarbonate (DEPC), ethidium bromide (EtBr), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) kits were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). The 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 were of reagent grades and locally or commercially available.

Animals

Male Sprague-Dawley rats, weighing 260-300 g, were supplied by the Jeil Animal Breeding Company, Korea, and were acclimatized to laboratory conditions at Sungkyunkwan University for at least one week, with food and tap water *ad libitum*.

Hepatic ischemia/reperfusion

After overnight fasting, the rats were anesthetized with pentobarbital sodium (35 mg/kg body weight, i.p.). The liver was exposed through a midline incision, and the pedicles of the left and median lobes occluded with a microvascular clamp for 90 min. This allowed 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 by the same procedure, but without placing the clamp in the left and median lobes. Six hours after 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.

Treatment with LPS and experimental groups

The animals were intraperitoneally injected with *Escherichia coli* endotoxin (LPS), at a dose of 1 mg/kg body wt., or saline, 1 mL/kg body wt. In the hepatic I/R group, the rats were intraperitoneally injected with LPS or saline at the time of reperfusion. All the rats were randomly assigned to the following four groups: (a) Sham (sham operation + saline injection), (b) LPS (sham operation + LPS injection), (c) I/R (I/R + saline injection) or (d) hepatic I/R + LPS.

Serum alanine and aspartate aminotransferase activity

The ALT and AST activities were determined by spectrophotometric procedures using the Sigma Diagnostics INFINITY™ kits 52-UV and 51-UV, respectively.

Total RNA extraction

Isolation of total RNA was carried out according to the method described by Chomczynski and Sacchi (1987). In brief, approximately 100 mg of liver tissue was obtained from the left lobe and 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, washed with 75% ethanol, then dissolved in DEPC-treated deionized water and stored at -70°C.

Reverse transcription and polymerase chain reaction (RT-PCR)

Reverse transcription of total RNA was performed to synthesize the first cDNA strand, using the oligo$(dT)_{12-18}$ primer and SuperScript II RNase H⁻ Reverse Transcriptase. The reverse transcriptase reaction was stopped by incubating it at 70°C for 10 min. The reaction products (cDNAs) were immediately stored at -20°C until the PCR analysis. PCR was carried out in 20 μL reaction volumes 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 and 0.5 U/

Table I. PCR primers used in the study

Gene (accession number)	Primer sequences (5' → 3')	Product length (bp)
ET-1 (M64711)	sense : TCTTCTCTCTGCTGTTTGTGGCTT anti-sense : TCTTTTACGCCTTTCTGCATGGTA	407
ET_A (M60786)	sense : AGTGCTAATCTAAGCAGCCAC anti-sense : CAGGAAGCCACTGCTCTGTAC	491
ET_B (X57764)	sense : AGCTGGTGCCCTTCATACAGAAGGC anti-sense : TGCACACCTTTCCGCAAGCACG	919
eNOS (AF085195)	sense : TGGGCAGCATCACCTACGATA anti-sense : GGAACCACTCCTTTTGATCGAGTTAT	202
iNOS (D44591)	sense : TTCTTTGCTTCTGTGCTTAATGCG anti-sense : GTTGTTGCTGAACTTCCAATCGT	1061
HO-1 (X13356)	sense : AAGGAGTTTCACATCCTTGCA anti-sense : ATGTTGAGCAGGAAGGCGGTC	568
COX-2 (U03389)	sense : CTGCATGTGGCTGATGTCATC anti-sense : AGGACCCGTCATCTCCAGGGTAATC	474
TNF-α (X66539)	sense : GTAGCCCACGTCGTAGCAAA anti-sense : CCCTTCTCCAGCTGGAAGAC	346
β-Actin (BC063166)	sense : TTGTAACCAACTGGGACGATATGG anti-sense : GATCTTGATCTTCATGGTGCTAG	764

reaction Ex *Taq*® DNA polymerase. The PCR amplification cycling (denaturation, annealing, extension) conditions were as follows: 94°C for 45 s, 65°C for 45 s and 73°C for 60 s, with 30 cycles for ET-1 and eNOS; 94°C for 45 s, 65°C for 45 s and 73°C for 60 s, 26 cycles for ET_A, ET_B and HO-1; 94°C for 45 s, 65°C for 45 s and 73°C for 60 s, 32 cycles for iNOS and 40 cycles for COX-2; 94°C for 30 s, 56°C for 30 s and 72°C for 60 s, 30 cycles for TNF-α; 94°C for 30 s, 62°C for 30 s and 72°C for 60 s, 25 cycles for β-actin. All PCR reactions had an initial denaturation step at 94°C for 5 min, and a final extension at 72°C for 5 min. To validate the RT-PCR results, β-actin specific primers were used in the PCR as a housekeeping gene.

RT-PCR product detection and densitometric analysis

Following RT-PCR, 10 μL aliquots 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 ± SEM. A one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test was used to determine the statistically significance differences between the experimental groups. The results were considered significant with a $P<0.05$.

RESULTS

Serum ALT and AST activity

The serum ALT and AST levels in the sham-operated group were 40 ± 6 and 156 ± 22 U/L, respectively. Although no apparent changes in the ALT and AST levels were seen with LPS alone, the ALT and AST levels were significantly increased by I/R alone. I/R + LPS resulted in greater significant releases of ALT and AST compared to I/R alone (Table II).

Vasoconstrictor genes expression

The level of ET-1 mRNA was significantly increased by LPS alone and I/R alone compared to the sham. No significant difference was detected between the I/R alone and I/R + LPS groups. There were no significant differences in the expression of ET_A receptor mRNA among the experimental groups. Similar to ET-1, the level of ET_B receptor mRNA was significantly increased in the LPS

Table II. Effect of LPS on serum aminotransferase activity after ischemia and subsequent reperfusion in rats

Groups	ALT (IU/L)	AST (IU/L)
Sham	40.1 ± 5.6	155.5 ± 22.4
LPS	77.8 ± 11.7	220.2 ± 32.6
I/R	1606.9 ± 204.4**	2946.7 ± 455.4**
I/R + LPS	2569.5 ± 182.5**,+	4587.0 ± 792.8**,+

Each value is the mean ± SEM for 8-10 rats per group. ** Significantly different from sham ($P<0.01$). + Significantly different from I/R ($P<0.05$). LPS, lipopolysaccharide; I/R, ischemia/reperfusion.

alone and I/R alone groups compared to the sham. No differences in the ET_B receptor transcripts were found between the I/R alone and I/R + LPS groups (Fig. 1).

Vasodilator genes expression

Although no apparent changes were seen in the level of eNOS mRNA in the LPS alone group compared to the sham, the expression of eNOS mRNA was significantly increased in the I/R alone compared to the sham, but even further increased in the LPS-treated I/R group. LPS resulted in a 7.5-fold increase in the level of iNOS mRNA in the sham-operated liver. I/R alone significantly induced the expression of iNOS. Furthermore, the expression of the iNOS gene was responsive to LPS treatment in the I/R group, resulting in significant increases in the mRNA levels compared to those with I/R alone. The HO-1 mRNA level was significantly increased by I/R, while no apparent changes were seen with LPS alone compared to the sham. Interestingly, LPS caused a marked increase of HO-1 transcripts in I/R livers, which was statistically different from I/R alone (Fig. 2).

TNF-α and COX-2 mRNA expression

I/R had no effect on the expression of TNF-α mRNA. LPS significantly increased the TNF-α transcripts in both

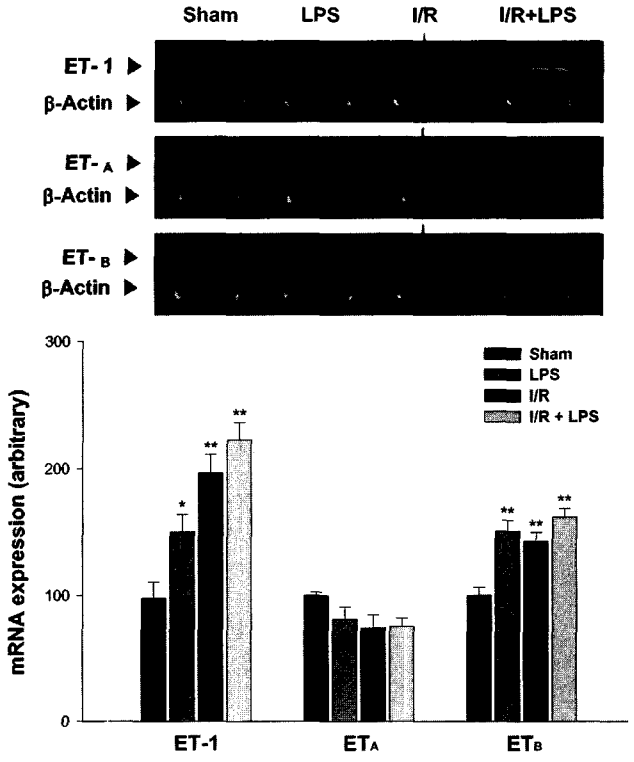

Fig. 1. Effect of LPS on the expressions of ET-1, ET_A and ET_B mRNA after ischemia and subsequent reperfusion in rats. Values are means ± SEM for 8-10 rats per group. *,** Significantly different from sham (P<0.05, P<0.01).

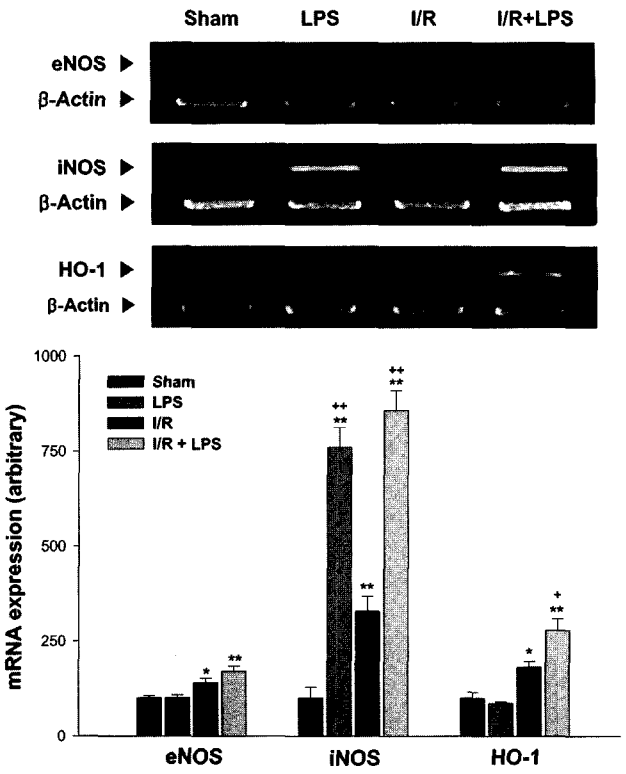

Fig. 2. Effect of LPS on the expressions of eNOS, iNOS and HO-1 mRNA after ischemia and subsequent reperfusion in rats. Values are means ± SEM for 8-10 rats per group. *,** Significantly different from sham (P<0.05, P<0.01). +Significantly different from I/R (P<0.05).

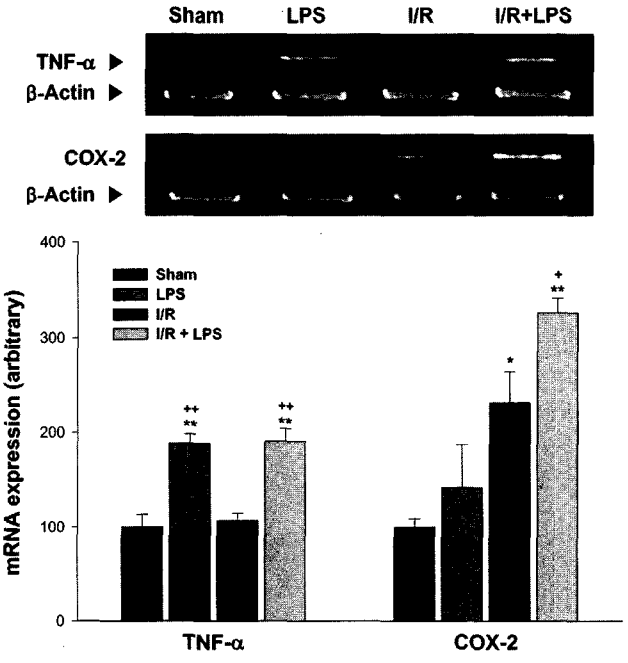

Fig. 3. Effect of LPS on the expressions of TNF-α and COX-2 mRNA after ischemia and subsequent reperfusion in rats. Values are means ± SEM for 8-10 rats per group. *,** Significantly different from sham (P<0.05, P<0.01). +,++ = Significantly different from I/R (P<0.05, P<0.01).

the sham-operated and I/R animals. There was a low level of COX-2 mRNA in the sham-operated group, but no significant difference was detected between the sham and LPS alone groups. However, I/R induced a marked increase in the COX-2 transcripts, and LPS treatment resulted in a further increase in these transcripts (Fig. 3).

DISCUSSION

I/R-induced hepatic injury is one of the major problems after transplantation of the liver (Thurman *et al.*, 1988). Functional and architectural changes in I/R predispose patients to secondary insults such as endotoxemia. A recent study by Colletti and Green (2001) showed that hepatic I/R in conjunction with LPS resulted in a more severe liver injury, and increased the levels of hepatic TNF-α, epithelial neutrophil activating protein and macrophage inflammatory protein-2 release. Nonetheless, the mechanisms of the increased susceptibility of liver I/R to endotoxemia remain largely unexplained.

Endotoxemia is a common complication of I/R, with a high probability of resultant multiple organ failure (Meakins, 1990). Microcirculatory dysfunction often proceeds to organ damage, and therefore, is considered a central component in hepatic failure. In the present study, the expressions of genes for various vasomodulators, which have been reported to play important roles in regulating the microcirculatory perfusion in the I/R liver and under conditions of I/R superimposed with endotoxemia, were determined. The dynamically balanced expressions of constrictive and dilatory forces are essential to maintain sinusoidal perfusion. The vasoactive mediators acting in the sinusoids on the constrictive side are endothelins, and those acting on the dilatory side are NO and CO, generated by NOS and HO, respectively (Pannen *et al.*, 1996).

In the present study, the serum ALT and AST levels were markedly increased after 6 h of reperfusion in ischemic livers. The greater increases in the plasma ALT and AST levels due to I/R + LPS demonstrated in this study are evidence confirming the exacerbation of hepatocellular injury in the double stressed model. Furthermore, this result indicates that the initiation of hepatic I/R prior to endotoxin challenge does not appear to have a tolerizing effect. In many situations an initial "priming" inflammatory event will have a tolerizing effect on the host for subsequent inflammatory stimuli, resulting in the production of lower levels of inflammatory mediators in response to the second inflammatory event. More specific to the I/R models, a dose of endotoxin given prior to the induction of renal I/R resulted in a decrease in renal injury (Heemann *et al.*, 2000). However, other models of hepatic injury, specifically bile duct ligation, also have a sensitizing effect on subsequent endotoxin challenge, again resulting in increased levels of inflammatory cytokines (Davoiskiba *et al.*, 2001). This suggests that several different signaling mechanisms occur in these differing models.

TNF-α has been implicated as an important mediator during inflammation, and has been shown to down-regulate the ET_A receptor gene expression during endotoxemia (Bucher and Taeger, 2002), whereas it up-regulates the prepro-ET-1 expression in various types of isolated cells (Nakano *et al.*, 1994). In addition, TNF-α has been shown to up-regulate the iNOS expression in mice from hemorrhage/resuscitation (Kahlke *et al.*, 2002). From our data, the TNF-α mRNA expression was increased in the I/R + LPS, but remained unchanged in the I/R alone animals. COX-2, the inducible isoform of cyclooxygenase, is primarily responsible for the synthesis of prostaglandins during stressful conditions. Dinchuk *et al.* (1995) showed that COX-2 mediates the endotoxin-induced liver injury in experiments with COX-2 deficient mice. In our data, the I/R group of animals demonstrated a marked increase in COX-2 mRNA expression, which was augmented by LPS treatment. These data indicate that TNF-α and COX-2 are important mediators in the development of the early phase of liver injury in the setting of endotoxemia following hepatic I/R.

Recent data provide some evidence that endothelium-derived vasoconstrictors and vasodilators may be functionally important in the control of total hepatic blood flow under I/R. Endothelin and its receptors are responsible for postischemic hepatic vasoconstriction and subsequent liver injury, which results from a lower oxygen and nutrient supply to hepatocytes (Rockey and Chung, 1998). In the present study, I/R alone and LPS alone increased the ET-1 mRNA expression. The I/R animals showed a trend towards an increase in ET-1 mRNA in response to LPS, but this increase failed to reach statistical significance compared to the I/R alone animals. Along with the increase in the ET-1 mRNA level, the gene expression of its receptor, ET_B, was significantly increased in the I/R + LPS group, but with no significant change in the ET_A transcripts. There were no significant differences in the ET_A and ET_B transcripts among the I/R alone, LPS alone and I/R + LPS groups. This finding was consistent with previous reports where there were increases in the ET_B mRNA transcript levels after I/R, while the ET_A transcript levels were slightly decreased (Yokoyama *et al.*, 2002). The increase in the ET_B receptor expression, coupled with no change in the expression of the ET_A receptors, would predict a predominant effect on the presinusoidal constrictor response where mediation of the ET_B receptor effects has been reported (Zhang *et al.*, 1994). The ET_B receptor message increased in response to LPS in the sham-operated animals, which was consistent with the previous reports of a higher proportion of the ET_B receptor during

endotoxemia (Bauer *et al.*, 2000). In this study, no ET_B receptor transcripts were found to be potentiated in the I/R + LPS group. This result indicates that a substantial portion of the constrictor effects of ET-1 may be mediated by ET_B receptors during hepatic I/R, which may not contribute to the dysfunction in the liver microcirculation upon a secondary stress.

To examine the counterbalancing forces to the pressor endothelin, the gene expressions of eNOS, iNOS and HO-1 were also determined. 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 an inflammatory response induces the expression of iNOS in the multiple cell types of the liver (Clemens *et al.*, 1994). Although increased levels of NO in peripheral vasculature have been suggested to be responsible for systemic hypotension and hyperdynamics, the bioavailability of NO is decreased in ischemic livers (Lefer and Lefer, 1999). Using different inhibitors of the NOS isozymes, several laboratories have demonstrated various effects of NOS inhibitors on I/R-induced liver injury ranging from protection to exacerbation of hepatocyte injury. No information is available on the regulation of heme oxygenase-mediated CO production in ischemic livers. The present study showed that the steady-state eNOS mRNA level was upregulated in I/R. Unlike sham-operated animals, the I/R animals showed an increase in the level of eNOS mRNA in response to LPS. The data of the current study revealed an LPS induced alteration in the iNOS gene expression in both the sham-operated and I/R livers. The hyperresponse of LPS-induced HO-1 gene expression seen in the I/R livers was of particular note despite the relatively unchanged levels of the HO-1 gene in the LPS alone animals. The mechanism underlying the sharpened increase in LPS-induced HO-1 gene expression in I/R is, however, unknown. Several recent studies have shown evidence that HO-1 is an inducible, cytoprotective enzyme, which is up-regulated under conditions of oxidative stress (Bulger *et al.*, 2003).

Taken collectively, the data from the present study showed an imbalanced gene expression of the hepatic vasoactive mediators in I/R challenged with endotoxemia, which favored vasodilation. The compromised ability of the I/R liver to counteract the increased pressor effects of ET-1, due to eNOS and HO-1, in response to endotoxemia may ultimately be responsible for hepatic microcirculatory dysfunction or failure. This could explain, at least partly, the susceptibility of the ischemic liver to endotoxemia.

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