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Role of Kupffer Cells in Vasoregulatory Gene Expression During Endotoxemia

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Abstract – Although hepatic microcirculatory dysfunction occurs during endotoxemia, the mechanism responsible for this remains unclear. Since Kupffer cells provide signals that regulate hepatic response in inflammation, this study was designed to investigate the role of Kupffer cells in the imbalance in the expression of vasoactive mediators. Endotoxemia was induced by intraperitoneal *E. coli* endotoxin (LPS, 1 mg/kg body weight). Kupffer cells were inactivated with gadolinium chloride ($GdCl_3$, 7.5 mg/kg body weight, intravenously) 2 days prior to LPS exposure. Liver samples were taken 6 h following LPS exposure for RT-PCR analysis of mRNA for genes of interest: endothelin (ET-1), its receptors ET_A and ET_B, inducible nitric oxide synthase (iNOS), heme oxygenase (HO-1), and tumor necrosis factor-α (TNF-α). mRNA levels for iNOS and TNF-α were significantly increased 31.8-fold and 26.7-fold in LPS-treated animals, respectively. This increase was markedly attenuated by $GdCl_3$, HO-1 expression significantly increased in LPS-treated animals, with no significant difference between saline and $GdCl_3$ groups. ET-1 was increased by LPS. mRNA levels for ET_A receptor showed no change, whereas ET_B transcripts increased in LPS-treated animals. The increase in ET_B transcripts was potentiated by $GdCl_3$. We conclude that activation of Kupffer cells plays an important role in the imbalanced hepatic vasoregulatory gene expression induced by endotoxin.

Keywords: Liver microcirculation, Endotoxemia, Vasomediators, Proinflammatory mediators.

INTRODUCTION

Impaired liver function is a common feature of clinical infectious illness. Clinical data have shown that the occurrence and degree of liver dysfunction greatly influence both the severity and the resolution of sepsis-induced multiple organ dysfunction syndrome (Hawker, 1991). Severe endotoxemia, induced either by the release of gram-negative bacterial endotoxins (lipopolysaccharides) or following the experimental exogenous administration of endotoxins, is a pathophysiological phenomenon accompanied by extended alterations in serum parameters and structural damage in various target organs. Although endotoxemia has been shown to cause hepatic microcirculatory failure and subsequent liver injury (Chun *et al*., 1994), the precise mechanism of endotoxemia-induced liver injury is not completely understood.

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Liver microcirculation is normally maintained through the fine balance of vasoconstrictors and vasodilators, of which endothelin-1 (ET-1), nitric oxide (NO), and carbon monoxide (CO) are the prominent vasomediators (Pannen *et al*., 1996). There is evidence that changes in the transcripts of genes related to vascular mediators in rat liver occur in response to an acute dose of endotoxin (Sonin *et al*., 1999).

Kupffer cells have been implicated in responses to endotoxin (Brown *et al*., 1997), burns (Wu et al., 1995), ischemia/reperfusion (Bradham *et al*., 1997), and sepsis (Bradham *et al*., 1997). In our studies, we employed gadolinium chloride ($GdCl_3$) to destroy Kupffer cells, based on the findings of other investigators showing the destruction of Kupffer cells following intravenous administration of this rare earth metal, a member of the trivalent cations of the lanthanide series (Koudstaal *et al*., 1991).

Large Kupffer cells were no longer present 24 h after $GdCl_3$ treatment (Hardonk *et al*., 1992). Splenic macrophages are less vulnerable to $GdCl_3$ because only source of the red pulp macrophages transiently disappear. The

white pulp macrophages are not affected. Interestingly, inactivation of Kupffer cells with $GdCl_3$ decreased the mortality of endotoxemia (Huynh *et al*., 2000) and prevented hepatocyte apoptosis and liver injury induced by LPS. Despite numerous investigations, a clear pattern of Kupffer cells alteration after injury remains to be elucidated. Furthermore, no information is available on Kupffer cell–mediated microcirculatory disturbance during endotoxemia.

Therefore, we hypothesized that activated Kupffer cells are somehow involved in mechanisms of endotoxemia–induced liver injury. Accordingly, the aim of this study was to understand whether Kupffer cells are indeed involved in hepatic vasoregulatory dysfunction during endotoxemia.

MATERIALS AND METHODS

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*. All animals were treated humanely according to the Sungkyunkwan University Animal Care Committee guidelines.

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 with saline, 1 ml/kg wt. In order to deplete the Kupffer cells, gadolinium chloride ($GdCl_3$, 7.5 mg/kg body weight) was injected via the penile vein at 1 and 2 days prior to LPS treatment. The rats were randomly assigned to the following four groups: (a) control (saline injection), (b) $GdCl_3$, (c) LPS, (d) $GdCl_3$ + LPS.

Analytical procedures

The plasma nitrite and nitrate (NOx) content was measured to estimate the total level of NO production. The plasma was filtered through a 10kDa molecular mass cut-off filter in order to eliminate any proteins. Nitrate was converted to nitrite through nitrate reductase.

The total nitrite level was measured using a total NO assay kit (Cayman, Ann Arbor, MI, USA). The serum alanine aminotransferase (ALT) activity was determined using the standard spectrophotometric procedure with a ChemiLab ALT kit (IVD Lab, CO., Ltd., Suwon, Korea).

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

The total RNA was extracted from liver tissue using TRIzol® reagent (GibcoBRL, NY, USA) according to the manufacturer's instructions. First strand cDNA was synthesized through reverse transcription of the total RNA using oligo dT-adaptor primer and AMV Reverse Transcriptase. PCR amplification was performed with a diluted cDNA sample in a 20-μl reaction volume. The final reaction concentrations were: primers, 10 pmol; $MgCl_2$, 2.5 mM; dNTP mixture, 1 mM; 10× RNA PCR buffer, 1×; Ex Taq® DNA polymerase, 0.5 U/reaction.

All PCRs included an initial denaturation step at 94°C for 5 min and a final extension at 72°C for 5 min in the GeneAmp 2700 thermocycler (Perkin-Elmer, Inc., Waltham, MA, USA). The following PCR amplification cycling conditions (denaturation, annealing, and extension) were used: 30 cycles of 94°C for 45 s, 65°C for 45 s, and 73°C for 60 s for ET-1 and heme oxygenase-1 (HO-1), 26 cycles for ET_A and ET_B, 32 cycles for iNOS; 30 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 60 s for tumor necrosis factor (TNF)-α; 25 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 60 s for β-actin.

β-Actin was used as a housekeeping gene to ensure equal loading of cDNA from the control and experimental samples. The amplified products were resolved by electrophoresis in 1.5% agarose gel and stained with ethidium bromide (Sigma–Aldrich, St. Louis, MO, USA). The intensity of each PCR product was evaluated semi-quantitatively using a digital camera (DC120, Eastman Kodak, Rochester, NY, USA) and a densitometric scanning analysis program (1D Main, Advanced American Biotechnology, Fullerton, CA, USA).

Statistical analysis

All data are presented as means ± SEM. One-way analysis of variance (ANOVA) followed by a Student–Neuman–Keuls test was used to determine the significance of the differences between the experimental groups. A P value < 0.05 was considered significant.

RESULTS

Plasma NOx levels

As indicated in Fig. 1, the plasma levels of NOx in the vehicle-treated control group and the $GdCl_3$-treated group were 10.3 ± 1.4 μM and 15.8 ± 2.5 μM, respectively. The

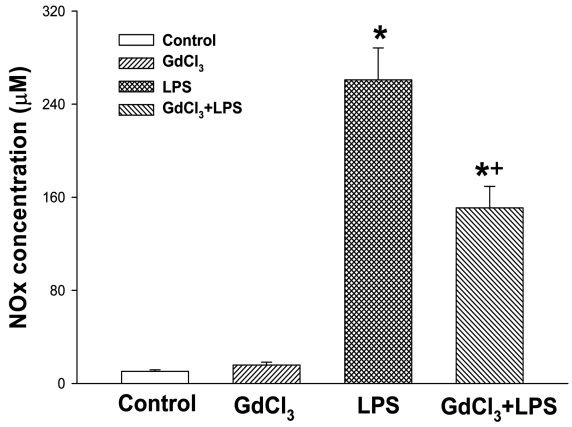

Fig. 1. Effect of gadolinium chloride on plasma NOx levels after LPS injection. Each bar represents the mean ± SEM for 7-8 rats per group. *P < 0.05 vs. control. ^{+}P < 0.05 vs. LPS.

plasma NOx level in the LPS group was significantly enhanced 25.3-fold compared with the vehicle-treated control group. This increase in NOx level was suppressed by $GdCl_3$ treatment before LPS injection (Fig. 1).

Serum ALT levels

The serum ALT levels in vehicle-treated control rats and $GdCl_3$-treated rats were 41 ± 3 U/L and 55 ± 4 U/L, respectively. In the LPS group, the ALT levels increased significantly compared with vehicle-treated control group. This increase in ALT level was attenuated by $GdCl_3$ pretreatment (Fig. 2). Similar to ALT levels, the serum AST levels increased to approximately 7.8 times that in the control animals and $GdCl_3$ significantly reduced this increase (data not shown).

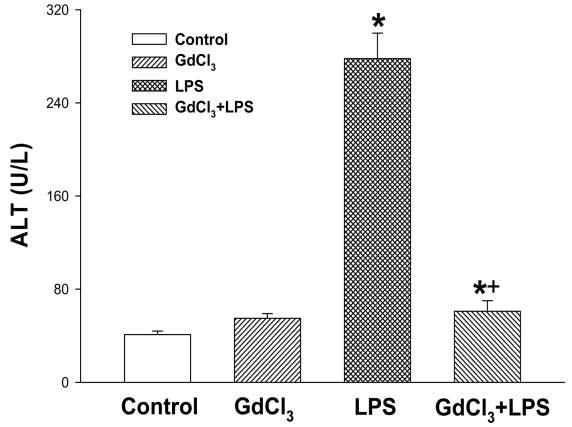

Fig. 2. Effect of gadolinium chloride on serum aminotransferase activity after LPS injection. Each bar represents the mean ± SEM for 7-8 rats per group. *P < 0.05 vs. control. ^{+}P < 0.05 vs. LPS.

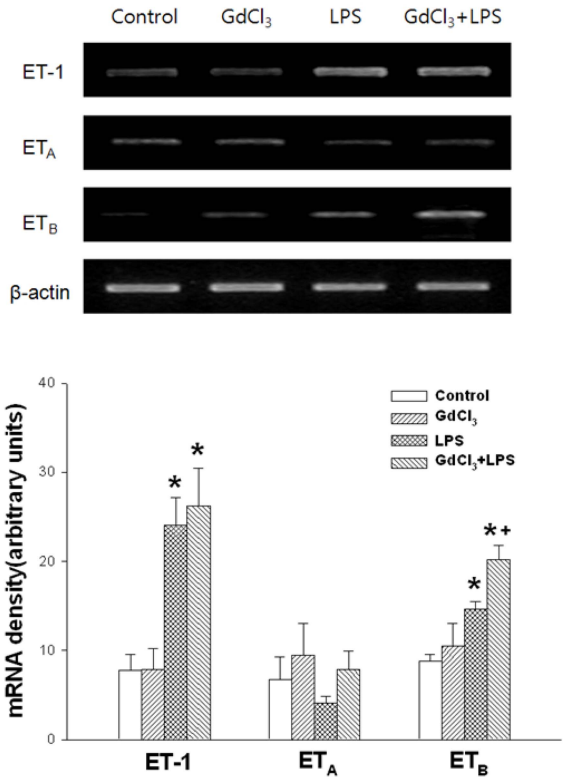

Fig. 3. Effect of gadolinium chloride on steady-state mRNA levels for genes of vasoconstrictors following endotoxemia. Each bar represents the mean ± SEM. *P < 0.05 vs. control. ^{+}P < 0.05 vs. LPS.

Role of Kupffer cells in expression of vasoactive mediators after LPS injection

The LPS group showed significantly higher expression levels of both ET-1 and the ET_B receptor mRNA transcript in liver compared with controls. Although pretreatment with $GdCl_3$ had no effect on the LPS-induced expression of ET-1 mRNA, the LPS-induced increase in ET_B receptor transcription was significantly enhanced by $GdCl_3$ pretreatment. There were no significant differences in the ET_A receptor mRNA levels among any of the experimental groups (Fig. 3). The iNOS level was significantly higher after LPS injection, and this increase was attenuated by $GdCl_3$. The level of HO-1 mRNA increased significantly in the LPS group. There were no significant differences in HO-1 mRNA levels between the LPS and the $GdCl_3$ + LPS groups (Fig. 4).

Role of Kupffer cells in expression of TNF-α after LPS injection

TNF-α is the most representative pro-inflammatory cytokine and is mainly released from Kupffer cells in liver.

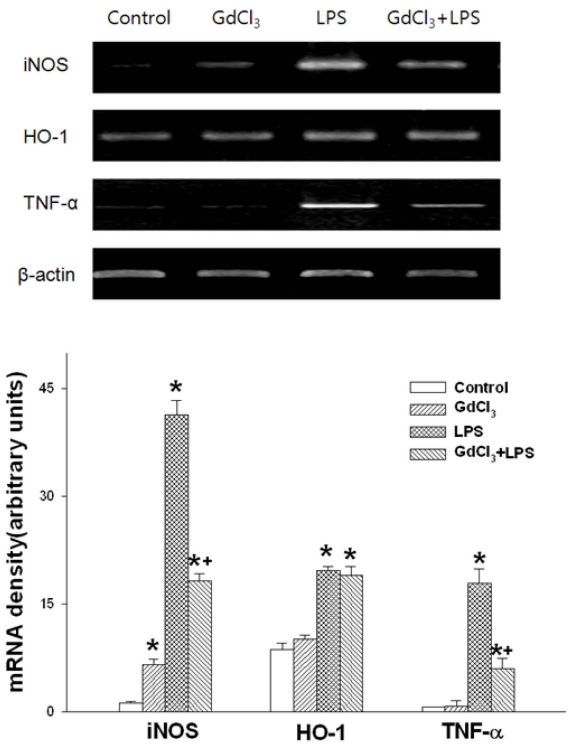

Fig. 4. Effect of gadolinium chloride on steady-state mRNA levels for genes of vasodilators and TNF-α following endotoxemia. Each bar represents the mean ± SEM. **P* < 0.05 vs. control. ^{+}P < 0.05 vs. LPS.

Thus we measured TNF-α mRNA expression level to confirm inflammatory status after LPS administration. The TNF-α-specific PCR band was barely detectable in control rat livers, and there was no increase after $GdCl_3$ alone. However, the TNF-α mRNA level was increased significantly by 26.7-times after LPS injection compared to the control group. This increase was significantly attenuated by $GdCl_3$ pretreatment (Fig. 4).

DISCUSSION

The degree of liver injury caused by I/R, endotoxemia, or other stress conditions is determined at least in part by impaired microcirculation (Chun *et al*., 1994).

Microcirculatory disturbances in the liver under stress are indicated by heterogeneity of perfusion (Clemens *et al*., 1994), narrowing of sinusoids, and increased contractility in response to vasoconstrictors (Clemens *et al*., 1994). Previous studies have shown that constriction of the sinusoids is mediated by hepatic stellate cells (Clemens *et al*., 1994). These cells actively regulate sinusoidal blood flow such that changes in their state, caused by stress factors, will affect microcirculation.

In our study we used a moderate stress model: 6 h after 1 mg/kg LPS injection for endotoxemia. These conditions do not cause an acute shutdown or sudden increase in permeability, but rather induce changes in vasculature responsiveness that are detected as increased contractility of sinusoid (Clemens *et al*., 1994) or sinusoid narrowing (Bauer *et al*., 1994; Okumura *et al*., 1994). The liver vascular response to stress depends largely on the level of the vasoactive mediator ET-1, its receptors A and B, and the vasodilators nitric oxide (NO) and carbon monoxide (CO) (Clemens *et al*., 1997).

The present data show an approximately 3-fold increase in ET-1 mRNA levels in endotoxemic rats. Increased ET-1 is one of the factors leading to microvascular impairment in shock (Pannen *et al*., 1996). There have been attempts to reduce liver injury by applying anti-ET-1 antibody or ET-blockers before the stress (Kitayama *et al*., 1997). Because Kupffer cell inactivation in conjunction with enhanced ET-1 levels during systemic stress may potentiate the hepatic microcirculatory dysfunction that ultimately leads to liver failure (Weitzberg *et al*., 1991), Kupffer cell ablation may mitigate ET-1 gene expression. In our study, pretreatment of animals with $GdCl_3$ did not influence ET-1 mRNA expression levels, suggesting that Kupffer cells are not directly involved in the transcriptional regulation of ET-1 during endotoxemia.

Previous studies have shown that both ET_A and ET_B agonists may increase portal vascular resistance in liver, and the ET_B receptor may be divided into two putative receptors, ET_{B1} and ET_{B2}. While ET_A and ET_{B2} receptors clearly mediate the profound sinusoidal contractile response, the role of ET_{B1} receptors in this context remains controversial. Recent studies indicate that the putative ET_{B1} receptors mediate the constriction of the hepatic sinusoids, but the effect tends to be masked by the concomitant vasodilatory actions of NO (Higuchi and Satoh, 1997). Along with the increase in ET-1 mRNA, expression of its receptor, ET_B, was significantly increased in endotoxemia, which is consistent with other studies that reported a higher ET_B receptor proportion during endotoxemia (Baveja *et al*., 2002). The increase in ET_B receptor expression was augmented by $GdCl_3$. There were similar levels of ET_A mRNA in all experimental groups. This suggests that a substantial portion of the constrictor effects of ET-1 may be mediated by the ET_B receptors and that the ET_B receptors may play an important role in the altered hepatic hemodynamics that occur during endotoxemia through a NO-dependent mechanism. LPS inhibited ET-1-induced eNOS activation in hepatic sinusoidal cells through a negative feedback

involving caveolin-1 (Kamoun el al., 2006). Interestingly, immunofluorescence analysis of isolated nonparenchymal cells revealed that Kupffer cells contained the eNOS inhibitory protein caveolin-1 (Calvo *et al*., 2001).

NO and CO are strong vasodilators (Pannen *et al*., 1996; Voerman *et al*., 1992) with a similar mechanism of action that involves activation of soluble guanylyl cyclase and enhanced cGMP production that leads to relaxation of hepatic stellate cells (HSC). Recent studies suggest that NO and CO most likely act in concert. Both NO and CO are produced constitutively, and both can be made by inducible enzymes upon stimulation. In intact liver under basal conditions, NO is produced by eNOS in sinusoidal endothelial cells (Shah *et al*., 1997). LPS is a potent inducer of iNOS in multiple cell types in the liver, most of all in Kupffer cells, but also in hepatocytes and HSC (Fleming *et al*., 1991). In our experiment, we observed ~32-fold increase in iNOS mRNA expression in total liver of endotoxemic rats. Kupffer cells affect the formation of NO as a result of iNOS overexpression. This is supported by the observation that Kupffer cell ablation via $GdCl_3$ pretreatment dramatically reduced the iNOS mRNA expression in the endotoxemic animals.

Another vasodilator CO is made constitutively from heme by HO-2 (Bauer *et al*., 1998; Goda *et al*., 1998). Additional CO can be produced by the inducible enzyme HO-1(HSP-32) upon stimulation. HO-2 is expressed in all liver cell fractions, whereas HO-1 was restricted to Kupffer cells in the normal rat liver. HO-1 gene expression is, however, inducible in hepatocytes and HepG2 cells in vivo and in vitro under conditions associated with oxidative stress (Bauer *et al*., 1998). Our data shows that HO-1 mRNA is highly induced in endotoxemic rats. These results are consistent with previous findings that inhibition of HO-1 upon LPS stimulation significantly increases vascular resistance (Bauer et al., 1996). No change in the level of HO-1 mRNA was observed in LPS groups compared with $GdCl_3$+LPS groups. Therefore, based on the present data, we cannot exclude the participation of other factors in the expression of HO-1 mRNA in liver from rats studied 6 h after LPS injection.

In summary, endotoxemia caused overt imbalanced gene expression of hepatic vasoactive mediators. Our findings suggest that activation of Kupffer cells is required for endotoxemia–induced hepatic microvascular dysfunction.

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