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Hepatic Vascular Stress Gene Expression in the Liver Response to Trauma

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Abstract – Trauma remains one of the important sources leading to systemic inflammatory response and subsequent multiple organ failure. Although hepatic microvascular dysfunction occurs during trauma, the mechanism responsible remains unclear. The aim of this study was to investigate the effect of trauma on hepatic vascular stress gene expression. Femur fracture (FFx) was induced by torsion to the femur at midshaft. Liver samples were taken for RT-PCR analysis of mRNA for genes of interest: endothelin-1 (ET-1), its receptors ET_A and ET_B, nitric oxide synthases (iNOS and eNOS), cyclooxygenase-2 (COX-2), heme oxygenase-1 (HO-1), and tumor necrosis factor-α (TNF-α). The expression of ET-1 mRNA was significantly increased by FFx. Expression of mRNA in FFx group showed no change in ET_A, ET_B, iNOS and HO-1 and showed a slight increase of 2.2-fold and 2.7-fold for eNOS and COX-2, respectively. The level of TNF-α mRNA significantly increased in FFx group. In conclusion, mild trauma alone causes little change in expression of vasoactive mediators.

Keywords □ femur fracture, liver microcirculation, vasoactive mediators, inflammatory mediators

INTRODUCTION

Although systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) continue to plague critically ill and injured surgical patients with a mortality of 50-80%, the mechanism and available treatment of the sequential injury have not been clearly identified (Deitch, 1992). SIRS can be seen in many conditions such as trauma, pancreatitis, burns, infection or major elective surgery (Bone, 1992). Trauma remains one of the important sources leading to SIRS and subsequent multiple organ failure (MOF), and this remote organ injury is mainly associated with the immunologic dissonance of patients themselves (Bone, 1996).

Liver failure is one of the hallmarks of MODS. To study the effect of trauma on remote organ injury, Schirmer *et al.* (1988) developed a femur fracture model and showed that blunt fracture caused a sustained and pathologic reduction in hepatic perfusion. A variety of mediators are thought to contribute to alterations of microvascular tone and blood flow during systemic inflammation (Chou *et al.*, 1995; Pscheidl *et al.*, 1994). Liver microcirculation is normally maintained under the fine balance of vasoconstrictors and vasodilators, of which endothelin-1 (ET-1), nitric oxide (NO), and carbon monoxide(CO) have been reported as the prominent vasomediators (Pannen *et al.*, 1996; Pannen *et al.*, 1998). Although we recently reported the changes in transcripts of the genes related to the vascular mediators in the rat liver to ischemia and reperfusion (Kim and Lee, 2004), expression of the genes encoding for proteins involved in vascular regulation remains largely undefined in trauma.

ET-1 is a potent vasoactive peptide that acts on its receptor types A and B (ET_A and ET_B, respectively) to mediate its action. ET_A receptor mediates constrictive actions of ET-1 although ET_B potentially can mediate both dilation and constriction, but in the hepatic portal circulation was reported to mediate constriction only (Yokoyama *et al.*, 2000). In intact liver, the constricting action of endothelin is in a balance with dilating action of nitric oxide and carbon monoxide, made constitutively by eNOS and heme oxygenase-2 (HO-2), respectively. The production of these vasodilators increases in the iver under certain stress conditions as a result of a stimulation of the inducible enzyme iNOS and HO-1, which may substantially contribute to the development of microcirculatory dysfunction (Furchgott and Jothianandan, 1991). Similarly, expression of the inducible HO-1 gene has been shown to be increased in the liver under different stress-induced conditions, such as hypoxia, endotoxemia, and ethanol consumption (Applegate *et al.*,

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1991). Although iNOS in particular has been extensively studied, information about changes in vasoregulatory gene expression is still incomplete, and very little is known about relationships among these various mediators. In particular, the mechanism regulating vasoregulatory gene expression during trauma has not been investigated.

Therefore, the aim of the present study was to investigate the effect of FFx trauma on the expression of hepatic vasoregulatory genes.

MATERIALS AND METHODS

Materials

All chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless specified otherwise.

Femur fracture (FFx)

All investigations adhered to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health under a protocol approved by Sungkyunkwan University. Anesthesia was induced by inhalation of halothane into male Sprague-Dawley rats weighing 250 to 300 g and who were fed *ad libitum*. While anesthetized, rats were randomized to receive closed FFx with described (Schirmer *et al.*, 1988). Control rats received anesthesia and shaving only. Briefly, two large Kelly clamps were applied at the proximal and distal ends of the left femur, and a sufficient force was introduced to fracture the femur at midshaft. Blunt fracture was ascertained by palpation. After the procedure, normal saline (10 ml per 100 g of body weight) was given subcutaneously in the anterior abdominal wall. After 3 days, FFx and control animals were killed with an overdose of halothane inhalation.

Reverse transcription and polymerase chain reaction

Total RNA was isolated from ~100 mg of liver tissue using RNA-STAT (TEL-TEST, Inc. Friendswood, TX, USA), treated with 10 U/μL of RNAse-free DNAse (Boehringer Mannheim, Indianapolis, IN, USA) in 10 mM $MgCl_2$, 20 mM Tris HCl at 37 °C for 30 min, and purification of RNA was dissolved in deionized water and stored at -80°C. Prior to the RT-PCR step, RNA samples from experimental groups were standardized by intensity of ribosomal RNA bands in the RNA aliquots stained with ethidium bromide and electrophoresed in a 1.5% agarose gel. The first strand cDNA was synthesized from 2 μg total RNA. Aliquots containing 1 μL of 10 mM deoxynucleoside triphosphates, 4 μL of 5× first-strand buffer, 1 μL of 50 μM random hexamer, 1 μL of 0.1 M dithiothreitol and 200 U of reverse transcriptase (Gibco-Bethesda Research Laboratory, Gaithersburg, MD, USA) were added and incubated at 37°C for 1.5 hours. The reaction was stopped by incubation at 95°C for 3 min, and freezed overnight at -20°C. Reaction tubes were then centrifuged at 7,500 rpm for 15 min at 4°C. The supernatant was discarded and cDNA pellet was resuspended in 50 μL of deionized water and utilized in the PCR reaction. In the PCR reaction, a diluted

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
GAPDH (BC059110)	sense : TCCCTCAAGATTGTCAGCAA anti-sense : AGATCCACAACGGATACATT	309

cDNA sample was amplified with gene-specific primers (Table I), in a total volume of 25 μL. The final reaction concentration were: primers, 1 μM; $MgCl_2$, 1.5 mM; deoxynucleotide triphosphates, 800 μM; 10× PCR buffer; Taq DNA polymerase, 0.5 μU/reaction. PCR for ET-1, ET_A, ET_B, HO-1, iNOS and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed with 30 cycles (95°C, 45 s; 65°C, 45 s; 72°C, 1 min) with an initial incubation at 95°C for 3 min, and a final extension at 72°C for 5 min. Reaction conditions consisted of 38 cycles and 24 cycles for COX-2 and TNF-α of 95°C for 30 s denaturation, 54°C for 30 s annealing and 72°C for 1 min extension, respectively. To ensure the use of equal amounts of cDNA from control and experimental samples in PCR, the aliquots of the RT products were used in PCR with the primers for the housekeeping gene GAPDH. Following RT-PCR, 10 μL samples of amplified products were resolved by electrophoresis in 1.5% agarose gel, stained with ethidium bromide. The level of each PCR product was semiquantitatively evaluated using a digital camera and a densitometric analysis program (Kodak Digital Science KDS 1D20, Eastman Kodak, New Haven, CT, USA).

Identification of RT-PCR products

A single band of predicted site was consistently found in all RT-PCR reactions : 407-, 491-, 919-, 568-, 1061-, 202-, 346-, 474-, and 309-bp fragments for ET-1, ET_A, ET_B, HO-1, iNOS, eNOS, TNF-α, COX-2 and GAPDH specific primers, respectively. The identity of PCR fragments was further confirmed by applying restriction enzymes according to the known gene sequences. No amplification was seen in the control PCR containing no cDNA or in the control RT-PCR conducted without reverse transcriptase.

Statistical analysis

All data were expressed as means ± SEM. One-way analysis of variance (ANOVA) followed by Student-Newman-Keuls *post hoc* test was used to determine the significance of the difference between groups. Results were considered significant for a $P<0.05$.

RESULTS

Effects of FFx on steady-state mRNA levels of the vasoconstrictor genes

Changes in expression of vasoconstrictor genes, ET-1 and its receptors (ET_A and ET_B), after FFx are shown in Fig. 1. 3 days after FFx, the level of ET-1 mRNA significantly increased as compared with controls. There were no significant differences in ET_A receptor mRNA levels among any of the experimental groups. In FFx rats, no change was detected in ET_B receptor mRNA compared to control rats.

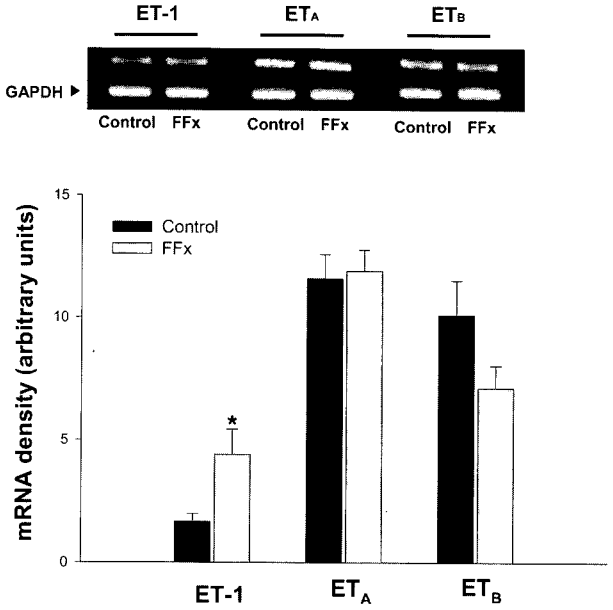

Fig. 1. Effect of FFx on steady-state of mRNA levels for genes of vasoconstrictors. Liver tissue samples were taken after 3 days after FFx. Effect of FFx on ET-1, ET_A, ET_B and GAPDH mRNA expression in the rat liver tissue was determined by semi-quantitative RT-PCR. Ethidium bromide-stained 1.5% agarose gel shows RT-PCR products made with total RNA from each group. For further details, see "Materials and Methods". Shown in A are representative bands from each group and shown in B are the densitometric analysis for transcripts of ET-1 (n=8 in each group), ET_A (n=7 in each group), and ET_B (n=7 in each group). Each bar represents mean ± SEM. *= Significantly different ($P < 0.05$) from control.

Effects of FFx on steady-state mRNA levels of the vasodilator genes

As shown in Fig. 2, there was a very low level of iNOS mRNA in control liver. FFx resulted in no significant increase in iNOS mRNA levels compared to controls. FFx showed a trend towards a slight increase in eNOS mRNA but failed to reach statistical significance compared to controls. No apparent changes in HO-1 mRNA level were seen in FFx compared to controls.

Effects of FFx on steady-state mRNA levels of COX-2 and TNF-α

The COX-2 specific PCR band was barely detectable in con-

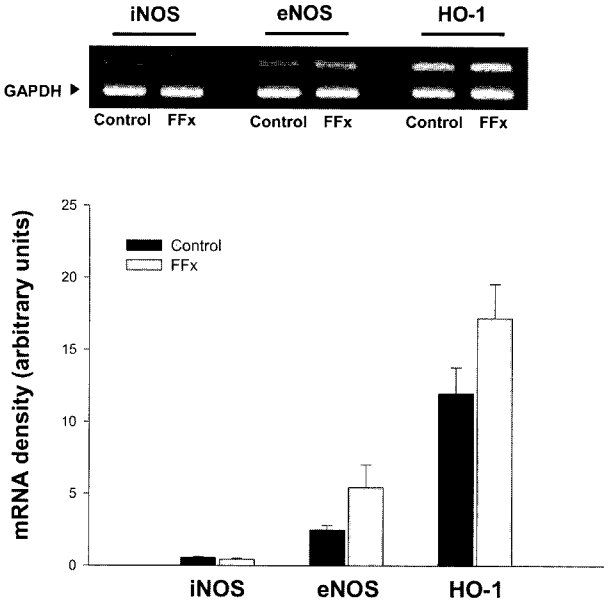

Fig. 2. Effect of FFx on steady-state of mRNA levels for genes of vasodilators. Liver tissue samples were taken after 3 days after FFx. For other details, see the legend for Fig. 1. Shown in A are representative bands from each group and shown in B are the densitometric analysis for transcripts of iNOS (n = 8 in each group), eNOS (n = 8 in each group), and HO-1 (n =8 in each group). Each bar represents mean ± SEM.

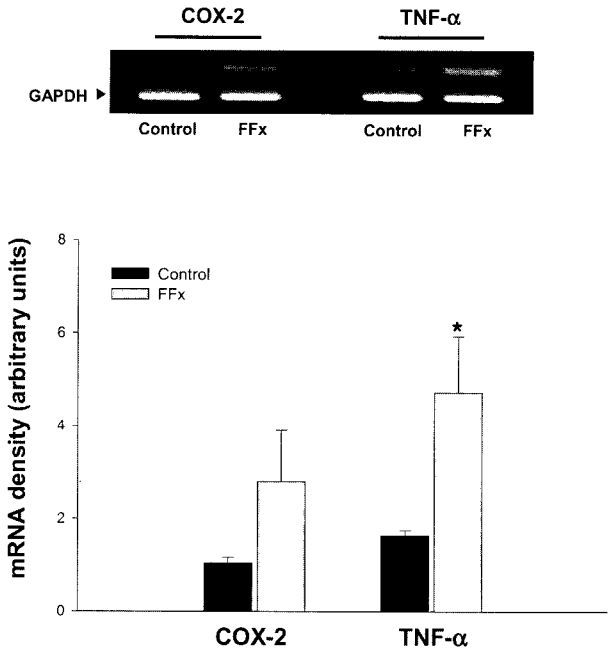

Fig. 3. Effect of FFx on steady-state of mRNA levels for COX-2 and TNF-α. Liver tissue samples were taken after 3 days after FFx. For other details, see the legend for Fig. 1. Shown in A are representative bands from each group and shown in B are the densitometric analysis for transcripts of COX-2 (n=8 in each group) and TNF-α (n=8 in each group). Each bar represents mean ± SEM. * = Significantly different ($P < 0.05$) from control.

trol rat liver, and blunt trauma showed a trend towards an increase in COX-2 mRNA but failed to reach statistical significance compared to controls. As with COX-2 gene expression, there was a low level of TNF-a mRNA in control liver. Expression of TNF-α mRNA significantly increased in FFx compared to controls (Fig. 3).

DISCUSSION

Trauma results in the activation of the hosts immune defense mechanisms to release a myriad of immunoactive cytokines and immune regulators. Although recent scientific advances have led to a better understanding of the immune system and the pathophysiologic response to inflammation (Napolitano and Campbell, 1995), the overwhelming clinical entity of trauma remains a controversial and multifactorial process. Studies have indicated that increased vascular permeability and vasodilation resulting from alterations in endothelial cells is an important mechanism underlying the pathophysiology of trauma. Schirmer *et al.* (1988) showed that blunt fracture caused a sustained and pathologic reduction in hepatic perfusion. When femur fracture was associated with soft tissue trauma, the elevated cardiac output was normalized at 48 hrs, but hpatic perfusion defect remained. However, the precise mechanism responsible for the hepatic microvascular response to trauma is not completely understood.

In the present study we determined the expression of genes for various vasomodulators that have been reported to play an important role in regulating the microcirculatory perfusion in the liver in oxidative stress and inflammatory reactions associated with stress. The dynamically balanced expression of constrictive forces and dilatory forces is essential to maintain sinusoidal perfusion. The vasoactive mediators acting in the sinusoids on the constrictive side are endothelins, and one that act on the dilatory side are NO and CO, generated by NOS and HO, respectively. Although posttranscriptional modifications can occur, previous studies have shown that vasomediators are primarily controlled at the level of transcription (Bauer *et al.*, 1998). Our results showed blunt trauma significantly increased ET-1 mRNA expression when compared to controls. This finding is consistent with previous reports showing markedly increased levels of ET-1 expression of endotoxemic rats (Voerman *et al.*, 1992). Increased concentration of ET-1 is one of the factors leading to microvascular impairment in shock (Pannen

et al., 1996). There were no significant differences in levels of ET_A and ET_B mRNA among any of the experimental groups.

If a change in receptor expression were to be responsible for the increase in response to endothelin in this model, we would predict that endothelin receptor numbers would be increased. In particular, we have previously reported that ET_A receptors mediate sinusoidal constriction as well as causing an increase in total portal resistance while ET_B receptors specially mediate an increase in total portal resistance without affecting sinusoid diameter (Kim and Lee, 2004). Contrary to what would have been predicted, the expression of ET_A and ET_B receptor remained unchanged. We postulate that this may be coupled with an increase in the production of functional receptors rather than expression of mRNA.

To examine the counterbalancing forces to the pressor endothelin, we also determined the gene expression of iNOS, eNOS, and HO-1. NO and CO are strong vasodilators and most likely act in concert. Both NO and CO are produced constitutively, and both can be made by inducible enzymes on stimulation. In intact liver under basal conditions, NO is produced by eNOS in sinusoidal endothelial cells. LPS is a potent inducer of iNOS in multiple cell types in the liver, most of all, in Kupffer cells, and also in hepatocytes and hepatic stellate cells (Taylor *et al.*, 1998). In our experiments, there was no difference in the levels of eNOS and iNOS mRNA between FFx animals and control animals. Another vasodilator CO is constitutively made from heme by HO-2 in all types of liver cells. Additional CO can be produced by the inducible enzyme HO-1 (HSP-32) on stimulation. Various conditions are able to induce HO-1 gene expression in the liver, *e.g.* hemorrhagic shock, endotoxemia, or glutathione depletion, however, with species, specific differences and a distinct acinar and cellular expression pattern (Paxian *et al.*, 2001). In our experiment, we found no change in the level of HO-1 mRNA in FFx rats compared with control rats.

TNF-α has been implicated as an important mediator of inflammation. Some studies indicate that TNF-α has a protective role against the lethal effects of lipopolysaccharide (LPS) (Freudenberg *et al.*, 1986). TNF-α has also been implicated in the effects of LPS on vasoregulation. TNF-α has been shown to downregulate ET_A receptor gene expression during endotoxemia, causing an attenuation of ET_A receptor mediated vascular response (Bucher and Taeger, 2002). Conversely, TNF-α has been shown to upregulate preproET-1 expression in various types of isolated cells (Nakano *et al.*, 1994). In addition, TNF-α has been shown to upregulate iNOS expression in mice from hemorrhage/resuscitation and induces HO mRNA in LPS-treated mice (Rizzardini *et al.*, 1993). However, the presence and regulation of TNF-α have not been previously documented in trauma. Trauma significantly increased mRNA expression of TNF-α. Although the exact etiology of multiorgan system failure caused by trauma remains unclear and may be multifactorial, our data led us to believe that TNF-α appears to be important in the pathogenesis of the trauma.

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 endotoxin-induced liver injury in experiments with COX-2 deficient mice. COX-2 gene, generally absent in resting cells, is rapidly induced by hormones, cytokines, and tumor promoters. In the context of liver fibrosis, ET-1 via its ET_B receptors and TNF-α induce NF-κB-dependent upregulation of COX-2, resulting in inhibition of hepatic stellate cells proliferation (Gallois *et al.*, 1998). In this study, all FFx animals showed a trend towards an increase in COX-2 mRNA but failed to statistical significance compared to controls.

In summary, our present results demonstrate that mild trauma causes little overt imbalanced gene expression of hepatic vasoactive mediators. However, a little imbalanced production of vasoactive mediators will result in "priming" of the vasculatures and greater damage in case of the second stress. Further studies of changes induced by stress in different types of the liver cells are required for better understanding of pathways leading to balanced or unbalanced expression of vasoactive mediators in the liver vasculature.

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