Arch Pharm Res Vol 26, No 12, 1079-1086, 2003

Archives of Pharmacal Research
http://apr.psk.or.kr

Differential Regulation of Cytochrome P450 Isozyme mRNAs and Proteins by Femur Fracture Trauma

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(Received August 25, 2003)

The aim of this study was to investigate the effect of trauma on cytochrome P450 (CYP) gene expression and to determine the role of Kupffer cells in trauma-induced alteration of CYP isozymes. Rats underwent closed femur fracture (FFx) with associated soft-tissue injury under anesthesia. To deplete Kupffer cells *in vivo*, gadolinium chloride ($GdCl_3$) was intravenously injected at 7.5 mg/kg body wt., 1 and 2 days prior to FFx surgery. At 72 h of FFx, liver tissues were isolated to determine the mRNA and protein expression of CYP isozymes and NADPH-P450 reductase by reverse transcription-polymerase chain reaction and Western immunoblotting, respectively. In addition, the mRNA levels of tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS) and heme oxygenase-1 (HO-1) were evaluated. FFx increased the mRNA level of CYP1A1; an increase that was not prevented by $GdCl_3$. There were no significant differences in the mRNA expression of CYP1A2, 2B1 and 2E1 among any of the experimental groups. The protein levels of CYP2B1 and 2E1 were significantly decreased by FFx; a decrease that was not prevented by $GdCl_3$ treatment. The gene expression of NADPH-P450 reductase was unchanged by FFx. FFx significantly increased the expression of TNF-α mRNA; an increase that was attenuated by $GdCl_3$. The mRNA expression of HO-1 was increased by FFx, but not by $GdCl_3$. Our findings suggest that FFx differentially regulates the expression of CYP isozyme through Kupffer cell-independent mechanisms.

Key words: Femur fracture, Cytochrome P450 gene expression, Kupffer cells

INTRODUCTION

Trauma remains one of the important sources leading to systemic inflammatory response syndrome (SIRS) and subsequent multiple organ failure (MOF), which are responsible for the high morbidity and mortality in the intensive care unit (ICU) (Brun-Buisson, 2000). Although SIRS and MOF are mainly associated with the immunologic dissonance of patients themselves, the mechanism and available treatment of the sequential injury has not been clearly identified (Bone, 1996).

Liver failure is one of the hallmarks of MOF. The activity of the liver in metabolizing and eliminating various drugs often decreases with infectious disease (Renton, 1986). In rodents suffering from bacterial infection or challenged with interleukin-1 (IL-1) and other inflammatory cytokines (Shedlofsky *et al.*, 1987), hepatic levels of cytochrome P450 (CYP) and drug-metabolizing activity in microsome both decreased. The hepatic CYP enzymes are composed of a large but closely related superfamily of distinct gene products with diverse substrate specificities (Nelson *et al.*, 1996). Our recent studies have shown that femur fracture (FFx) trauma causes mild alterations of hepatic CYP-dependent drug metabolism (Lee and Lee, 2003). Many previous reports also showed that CYP isoforms were differentially modulated by nitric oxide (NO) in endotoxemic rats after administration of lipopolysaccharide (LPS) (Takemura *et al.*, 1999).

Macrophages play a crucial role in regulating host defense mechanisms after trauma, shock and sepsis, and their activation initiates inflammatory responses to injury (Nielsen *et al.*, 1994). Kupffer cells constitute 80% of the fixed macrophages and reside at a strategic position in hepatic sinusoids where they interact with hepatocytes, other leukocytes and variable mediators. Previously, Kupffer

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cells were shown to mediate responses to endotoxemia (Brown *et al.*, 1997), burns (Wu *et al.*, 1995), ischemia/reperfusion (Bradham *et al.*, 1997) and sepsis (Koo *et al.*, 1999), and to regulate the synthesis of acute phase proteins by hepatocytes. Furthermore, Kupffer cells produce important inflammatory mediators, including tumor necrosis factor-α (TNF-α), superoxide, NO, prostaglandin E_2 (PGE_2) and other cytokines (Cutrin *et al.*, 1998). However, few studies have examined the direct effect of trauma on Kupffer cell function *in vivo*.

Therefore, the aim of the present study was to investigate the effect of FFx trauma on the expression of hepatic CYP isozyme genes, and to determine the role of Kupffer cells in altered gene expression.

MATERIALS AND METHODS

Chemicals

Gadolinium chloride ($GdCl_3$), sodium dodecyl sulfate (SDS), bis-acrylamide, ammonium persulfate, Tris-HCl, *N,N,N',N'*-tetramethylethylenediamine, ethidium bromide (EtBr) and *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Acrylamide, nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were supplied from Bio-Rad Lab. (Hercules, CA, USA). RNA PCR kit R019A (AMV) and Ex *Taq*® DNA polymerase were purchased from TaKaRa Schuzo Co. (Shiga, Japan). All other chemicals used were of reagent grades and were locally and commercially available.

Animals

Male Sprague-Dawley rats weighing 230 to 250 g (7 weeks of age) were supplied by 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*. The animals were kept in a temperature and humidity controlled room (25±1°C and 55±5%, respectively) with a 12 h light-dark cycle.

Femur fracture

Anesthesia was induced by the intraperitoneal injection of xylazine hydrochloride (20 mg/kg) and ketamine hydrochloride (50 mg/kg). While anesthetized, rats were randomized to receive closed FFx with associated soft-tissue injury as described by Schirmer *et al.* (1988). Control rats received anesthesia and shaving only. Shortly, two Kelly clamps were applied at the proximal and distal ends of the left femur, and sufficient torsion was supplied to fracture the femur midshaft. Blunt fracture was ascertained by palpation. After the procedure, sterilized physiological saline (10 mL/100 g of body wt.) was administered subcutaneously in the dorsal wall. Rats had access to food and water *ad libitum* during recovery from anesthesia. The whole liver was removed 72 h after FFx for experimentation.

Pretreatment with $GdCl_3$ and experimental groups

To deplete Kupffer cells *in vivo*, $GdCl_3$ (7.5 mg/kg/mL, dissolved in sterilized physiological saline) was injected *via* the tail vein, 1 and 2 days before FFx surgery. In the control rats, physiological saline was injected in the same volume and manner as for $GdCl_3$. Four experimental groups were studied: (a) vehicle-treated control, (b) $GdCl_3$-treated control, (c) FFx and (d) $GdCl_3$-treated FFx.

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

Isolation of total RNA was carried out according to the method described by Chomczynski and Sacchi (1987). Briefly, liver tissue was homogenized in the presence of TRIZOL® reagent (GibcoBRL, NY, USA). After extraction with chloroform, the total RNA was precipitated from the aqueous phase by addition of isopropanol, washed with 75% ethanol, solubilized with diethylpyrocarbonate-treated water and stored at -75°C. Reverse transcription of total RNA was performed to synthesize the first strand cDNA using oligo dT-adaptor primer and AMV Reverse Transcriptase (TaKaRa, Shiga, Japan). PCR reaction was performed with a diluted cDNA sample and amplified in each 20 μL reaction volume. The final reaction concentration were: primers, 10 pM; $MgCl_2$, 2.5 mM; dNTP mixture, 1 mM; 10X RNA PCR buffer, 1X; Ex *Taq*® DNA polymerase, 0.5 unit/reaction. The gene-specific primers used are listed in Table I. All PCR reactions had an initial denaturation

Table I. PCR primers used in the study

Gene	Primer sequences (5' → 3')	Product length (bp)
CYP1A1	sense : CTGGTTCTGGATACCCAGCTG anti-sense : CCTAGGGTTGGTTACCAGG	331
CYP1A2	sense : CAGTCACAACAGCCATCTTC anti-sense : CCACTGCTTCTCATCATGGT	302
CYP2B1	sense : TTGTTTGGTGCTGGGACAGAG anti-sense : GGCTAGGCCCTCTCCTGCACA	443
CYP2E1	sense : AAACTTCATGAAGAAATTGAC anti-sense : TCTCCAACACACACACGCTTTCC	311
TNF-α	sense : GTAGCCCACGTCGTAGCAA anti-sense : CCCTTCTCCAGCTGGAAGAC	346
iNOS	sense : TTCTTTGCTTCTGTGCTAATGCG anti-sense : GTTGTTGCTGAACTTCCAATCGT	1061
HO-1	sense : AAGGAGTTTCACATCCTTGCA anti-sense : ATGTTGAGCAGGAAGGCGGTC	568
NADPH-P450 reductase	sense : GGCTCCCAGACGGGAACCGCT anti-sense : CCTCCACGTGATGAAATCCTC	407
β-Actin	sense : TTGTAACCAACTGGGACGATATGG anti-sense : GATCTTGATCTTCATGGTGCTAG	764

step at 94°C for 5 min, and a final extension at 72°C for 5 min using a GeneAmp 2700 thermocycler (Perkin-Elmer, Norwalk, USA). The PCR amplification cycling conditions were as follows: 94°C 30 s, 56°C 30 s, 72°C 60 s, 28 cycles for TNF-α; 94°C 45 s, 65°C 45 s, 73°C 60 s, 30 cycles for inducible nitric oxide synthase (iNOS) and heme oxygenase-1 (HO-1); 94°C 30 s, 57°C 30 s, 72°C 60 s, 25 cycles for NADPH-P450 reductase and 32 cycles for CYP1A1; 94°C 30 s, 62°C 30 s, 72°C 60 s, 22 cycles for CYP1A2 and 23 cycles for CYP2B1; 94°C 30 s, 60°C 30 s, 72°C 60 s, 23 cycles for CYP2E1 and 25 cycles for β-actin. 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 β-actin. Following RT-PCR, amplified products were resolved by electrophoresis in 1.5% agarose gel, and stained with EtBr. The intensity of each PCR product was semiquantitatively evaluated using a digital camera (DC120, Eastman Kodak, CT, USA) and a densitometric scanning analysis program (1D Main, Advanced American Biotechnology, CA, USA).

Preparation of liver microsomes and Western blot immunoassay

The excised liver was minced and homogenized with a teflon pestle homogenizer in 4 volumes of homogenizing buffer containing 1.15% (w/v) KCl and 50 mM Tris-HCl (pH 7.4). The whole homogenate was centrifuged at 10,000 ×g for 60 min and then the supernatant was centrifuged at 105,000×g for 60 min at 4°C. Microsomal precipitates were resuspended with 10 volumes of 1.15% (w/v) KCl solution containing 10 mM HEPES and 1 mM EDTA, aliquoted and frozen at -75°C until assayed. The content of microsomal protein was determined using the Bio-Rad protein assay reagent with bovine serum albumin as a standard. Microsomal proteins (10 μg/well) from each sample were loaded on a 10% SDS-polyacrylamide gel and separated by electrophoresis. The separated proteins were transferred to nitrocellulose membranes by Semi-Dry Transfer System (Bio-Rad Lab., CA, USA). Bands were immunologically detected using polyclonal goat anti-rat CYP1A1, 2B1, 2E1 and NADPH-P450 reductase primary antibody (Gentest, MA, USA) and then incubated with an alkaline phosphatase conjugated monoclonal rabbit anti-goat IgG secondary antibody (Sigma Chemicals Co., MO, USA). The color of immune complexes was developed with a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. The intensity of the immuno-reactive bands was determined by scanning with the densitometric analysis program.

Statistical analysis

All data are presented as means±SEM. One-way analysis of variance (ANOVA) followed by Dunnett's *t*-test was used to determine the statistical significance of the differences between experimental groups. A *P* value <0.05 was deemed to be significant.

RESULTS

CYP isozyme mRNA expression

As shown in Fig. 1A, the level of CYP1A1 mRNA expression markedly increased after FFx; an increase which was not prevented by $GdCl_3$ pretreatment. FFx did not affect mRNA expression levels of CYP1A2, 2B1 or 2E1 (Figs. 2, 3A and 4A, respectively).

CYP isozyme protein expression

In Western blot immunoassay for CYP1A1, there was no remarkable change in the amount of protein expression

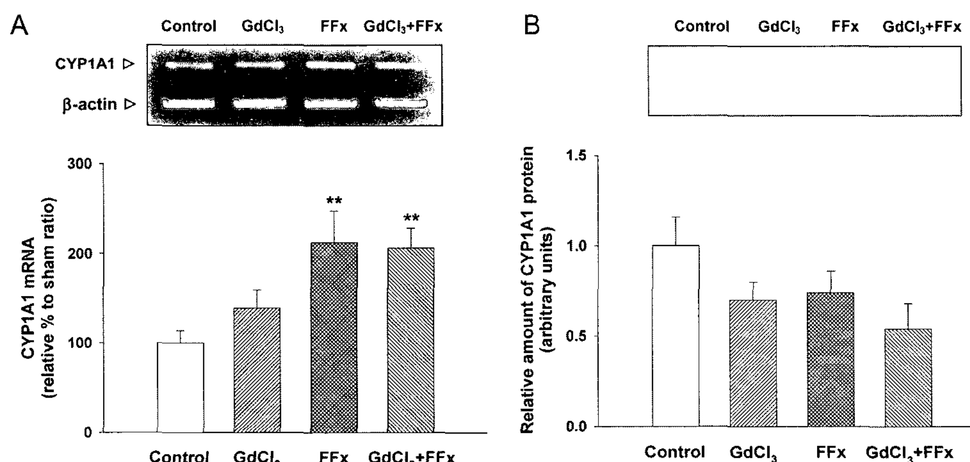

Fig. 1. Effect of $GdCl_3$ pretreatment on CYP1A1 mRNA (A) and protein (B) expression after FFx. **Significantly different (p<0.01) from controls. Values are means±SEM for 4-6 rats per group.

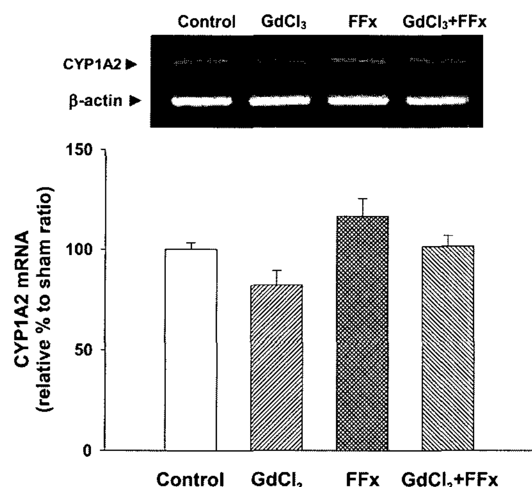

Fig. 2. Effect of $GdCl_3$ pretreatment on CYP1A2 mRNA expression after FFx. Values are means±SEM for 4-6 rats per group.

(Fig. 1B). However, the amount of CYP2B1 and 2E1 protein expression was markedly decreased 72 h after FFx (Figs. 3B and 4B, respectively). This decrease was not prevented by $GdCl_3$ pretreatment. $GdCl_3$ itself markedly decreased the amount of CYP2E1 isozyme protein expression.

Steady-state mRNA levels of TNF-α and iNOS

As shown in Fig. 5A, a low level of TNF-α mRNA was observed in the vehicle-treated control liver. However, TNF-α mRNA expression was significantly increased after FFx. This increase was prevented by $GdCl_3$ pretreatment. As with TNF-α gene expression, the iNOS specific PCR band was barely detectable in the control liver, and there was no notable increase after FFx (Fig. 5B).

Steady-state mRNA levels of heme oxygenase-1

The level of HO-1 mRNA in the control rat liver was

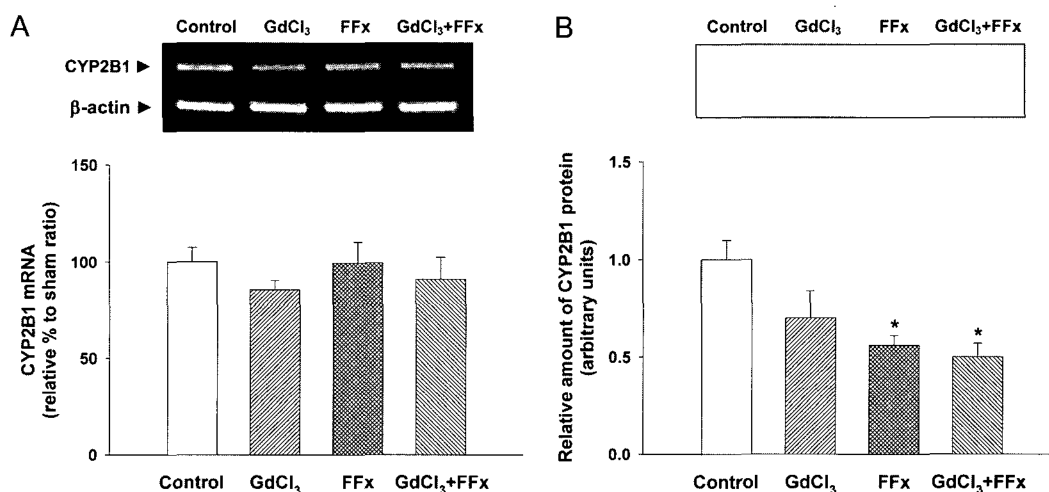

Fig. 3. Effect of $GdCl_3$ pretreatment on CYP2B1 mRNA (A) and protein (B) expression after FFx. *Significantly different ($p<0.05$) from controls. Values are means±SEM for 4-6 rats per group.

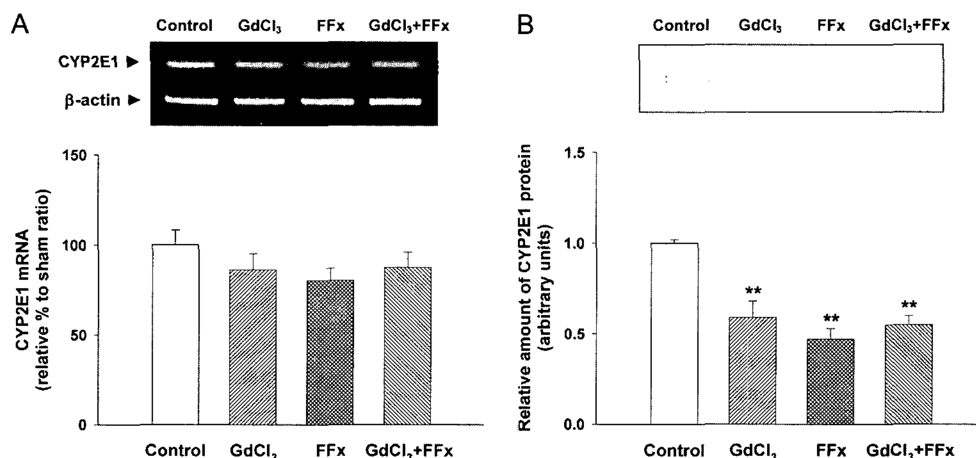

Fig. 4. Effect of $GdCl_3$ pretreatment on CYP2E1 mRNA (A) and protein (B) expression after FFx. **Significantly different ($p<0.01$) from controls. Values are means±SEM for 4-6 rats per group.

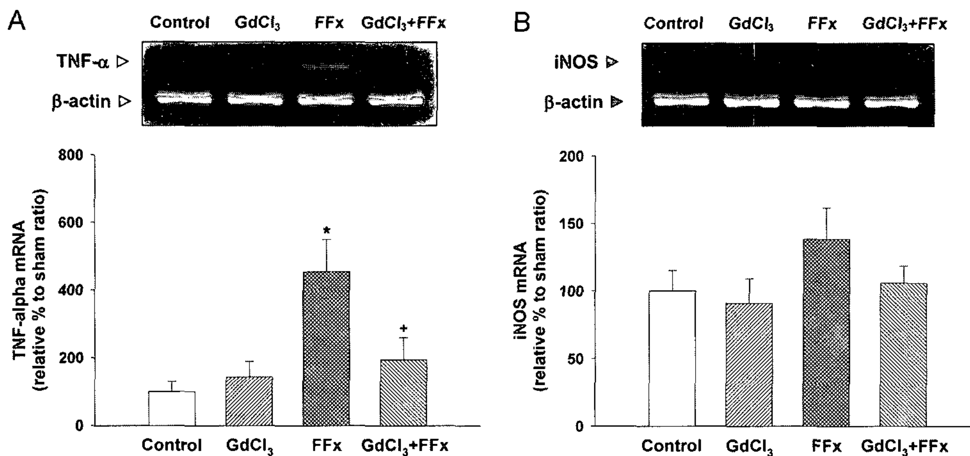

Fig. 5. Effect of $GdCl_3$ pretreatment on TNF-α (A) and iNOS (B) mRNA expression after FFx. *Significantly different ($p<0.05$) from controls. +Significantly different ($p<0.05$) from FFx group. Values are means±SEM for 4-6 rats per group.

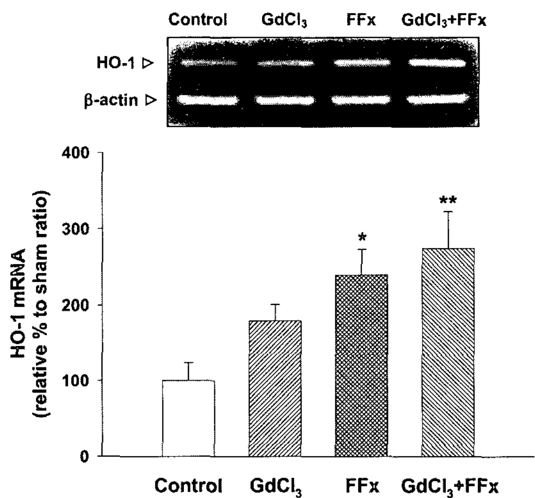

Fig. 6. Effect of $GdCl_3$ pretreatment on HO-1 mRNA after FFx. *,**Significantly different ($p<0.05$, $p<0.01$) from controls. Values are means±SEM for 4-6 rats per group.

rather high. There was also an increase in the expression of mRNA for HO-1 after FFx. $GdCl_3$ did not affect the increase in HO-1 mRNA (Fig. 6).

NADPH-P450 reductase mRNA and protein expression

As shown in Fig. 7, there were no significant changes in the mRNA and protein expression of NADPH-P450 reductase among any of the experimental groups.

DISCUSSION

The hepatic CYP-dependent mono-oxygenase system which is responsible for the metabolism of a number of endogenous and xenobiotic compounds is often depressed by agents that alter host defense mechanisms. The depression of this enzyme activity occurs in both animals and humans during active viral infection and causes a

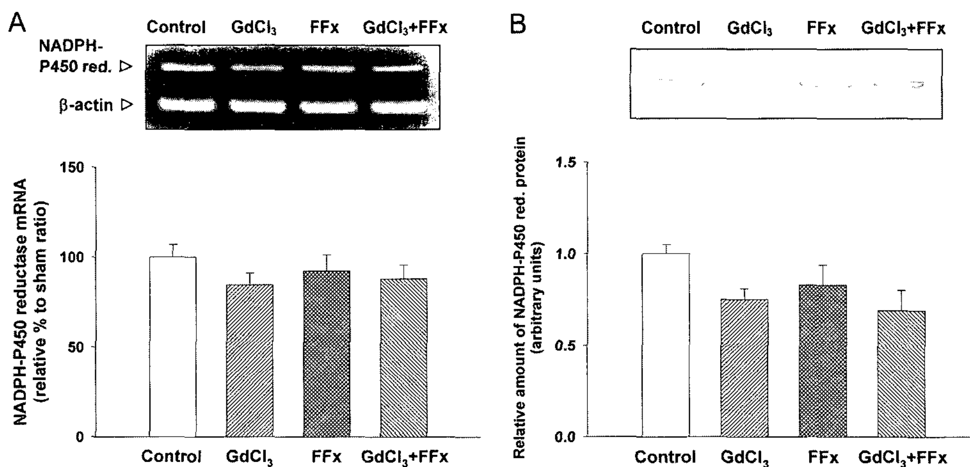

Fig. 7. Effect of $GdCl_3$ pretreatment on NADPH-P450 reductase mRNA (A) and protein (B) expression after FFx. Values are means±SEM for 4-6 rats per group.

decline in the capacity of the liver to eliminate drugs (Kraemer *et al.*, 1982). Other pathophysiological conditions, such as endotoxemia (Takemura *et al.*, 1999), inflammation (Iber *et al.*, 1999) and ischemia/reperfusion injury (Eum *et al.*, 2002) have also been reported to modulate CYP enzyme activity. The liver has high levels of CYP isoforms with different specificity for substrates. In our previous study, FFx decreased total liver microsomal CYP content and the activities of CYP2E1 (Lee and Lee, 2003). Regulation of CYP gene expression is governed by a number of mediating factors, such as the presence of inducing agents and/or altered endogenous cytokines (Denison and Whitlock, 1995). It would appear that the regulatory segments of CYP genes vary, even within the same gene family of subfamily, and this would explain why closely related CYP genes can differ in their regulation and expression (Kemper, 1993). In the present study, differential expression of CYP isozyme genes was observed in traumatic rats after FFx.

Isozymes that belong to the CYP2B subfamily have been extensively studied in many species, and CYP2B1/2B2 is known as the major phenobarbital-inducible CYP isozyme in rats. In the present study, the level of CYP2B1 mRNA was not altered but the amount of protein expression significantly decreased after FFx. This result indicates that trauma may regulate the expression of CYP2B1 at the post-transcriptional level. CYP2E1 is significant for its adaptive response to high blood ethanol levels with a corresponding acceleration of ethanol metabolism, and is inducible by small organic molecules and pathophysiological states (Hong *et al.*, 1987). Interestingly, LPS down-regulates hepatic expression of CYP2E1 (Sewer *et al.*, 1996). In our study, CYP2E1 protein expression, but not mRNA expression, was markedly decreased by FFx, indicating that FFx may suppress CYP2E1 translationally. CYP1A1 and 1A2 are known as isoenzymes that exist in almost all mammalian species. Although subtle differences exist between these two isozymes, their function, which plays an important role in xenobiotic metabolism as well as in carcinogenesis, is fairly well conserved across species. In contrast to CYP2B1 and 2E1, the level of CYP1A1 mRNA expression, but not of CYP1A2, was significantly increased after FFx. FFx enhances CYP1A1 mRNA, but there is little evidence for stimulation of CYP1A1 protein synthesis. This suggests that FFx may up-regulate the expression of CYP1A1 transcriptionally. Unfortunately we could not identify any band equivalent to the CYP1A2 enzyme size (59.3 kDa; data not shown).

TNF-α is thought to be a proximal mediator of the inflammatory response induced by LPS and most likely triggers the release of other cytokines. The administration of TNF-α has been shown in several studies to decrease total CYP as well as CYP1A, 2B, 2C, 2E and 3A subfamilies (Monshouwer *et al.*, 1996). In the present study, the mRNA level of TNF-α was markedly elevated by FFx, which suggests that TNF-α generated by FFx may be partially responsible for the regulation of CYP isozymes. It has been shown that the NO released following cytokine administration can inhibit CYP1A1 activity, protein and mRNA level (Stadler *et al.*, 1994). In contrast, Zamora *et al.* (2001) have shown that NO from *i*NOS increases the expression of CYP2E1 in iNOS-null hepatocytes. In our study, the mRNA level of iNOS was unchanged by FFx, indicating that NO is not responsible for the regulation of CYP isozyme by FFx. The idea that inflammatory mediators have differential effects on CYP regulation is supported by previous findings. Calleja *et al.* (1997) have shown that cytokines may differentially affect the gene expression of CYP isozymes in cultured rabbit hepatocytes.

Kupffer cells modulate the synthesis of acute phase proteins by hepatocytes and release powerful inflammatory mediators including TNF-α, NO, reactive oxygen species (ROS) and a variety of other cytokines (Cutrin *et al.*, 1998). Accordingly, they have been considered in several previous reports to play a primary role in the pathogenesis of liver damage (Caldwell-Kenkel *et al.*, 1991). Recently, it was reported that FFx trauma increased the phagocytosis and superoxide formation by Kupffer cells (Huynh *et al.*, 1997). Although Kupffer cells are responsible for liver injury due to variable pathophysiological conditions, several previous studies failed to show any ameliorating effect of the depletion of Kupffer cells on organ dysfunction (Reinders *et al.*, 1997), and therefore the role of Kupffer cells remains controversial. In the present study, FFx significantly increased TNF-α mRNA expression, and this increase was attenuated by $GdCl_3$. However, $GdCl_3$ pretreatment did not affect the expression of CYP isozyme mRNA and protein in FFx rats. Our results indicate that Kupffer cells play little role in the regulation of CYP expression after FFx, thereby implicating hepatocytes or other nonparenchymal cells.

Recently, Kumamoto *et al.* (1999) raised the possibility that hepatocytes could serve as a primary site of oxidant generation and thereby jeopardize the viability of adjacent nonparenchymal cells in a paracrine manner. Microsomal HO-1 is induced not only by its substrate (Alam *et al.*, 1989), but also by oxidative stress, and is thought to play an important protective role against oxidative injuries (Stocker, 1990). Previous studies revealed that induction of HO-1 occurred in the liver in response to burns (Nakae and Inaba, 2002), endotoxemia (Choi and Alam, 1996), hemorrhagic shock (Bauer *et al.*, 1996) and ischemia-reperfusion (Sonin *et al.*, 1999). It has also been reported that the heme of CYP isozyme was degraded to biliverdin by HO-1 (Kutty *et al.*, 1988). FFx significantly increased the level of HO-1 mRNA expression indicating that HO-1

may be another factor of CYP isozyme regulation. CYP-dependent oxidation requires NADPH-P450 reductase for electron transfer. In contrast to CYP isozymes, there was no significant change in mRNA and protein levels of NADPH-P450 reductase, indicating that FFx may regulate CYP protein (heme-containing protein), but not NADPH-P450 reductase (non-heme protein).

In summary, blunt FFx trauma differentially regulated CYP isoforms. Our findings suggest that the activation of Kupffer cell is not required for trauma-induced regulation of CYP isozyme expression.

ACKNOWLEDGEMENT

This work was supported by grant R04-2001-000-00011-0 from the Basic Research Program of the Korea Science and Engineering Foundation. o

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