

# Inflammatory prompts produce isoform-specific changes in the expression of leukotriene B<sub>4</sub> $\omega$ -hydroxylases in rat liver and kidney

Auinash Kalsotra<sup>a,1</sup>, Xiaoming Cui<sup>a,1,2</sup>, Leposava Antonovic<sup>a</sup>, Aaron M. Robida<sup>b</sup>,  
Edward T. Morgan<sup>b</sup>, Henry W. Strobel<sup>a,\*</sup>

<sup>a</sup>Department of Biochemistry and Molecular Biology, University of Texas Medical School at Houston, Houston, TX 77030, USA

<sup>b</sup>Department of Pharmacology, Emory University, Atlanta, GA 30322, USA

Received 2 October 2003; accepted 7 October 2003

First published online 3 November 2003

Edited by Barry Halliwell

**Abstract** Cytochrome P450 (CYP) 4Fs metabolize leukotriene B<sub>4</sub> and other inflammatory mediators in the arachidonic acid cascade. Here we show that lipopolysaccharide (LPS) treatment suppresses CYP4F4 and up-regulates CYP4F5 mRNA expression in rat liver whereas renal CYP4Fs are essentially unchanged. BaSO<sub>4</sub> treatment, in contrast, increases both hepatic and renal CYP4F expression levels. Thus, distinct regulatory mechanisms in CYP4F expression might operate under different inflammatory prompts. To examine hepatic totipotency, primary hepatocytes were treated with varying doses of LPS resulting in decrease in all the CYP4F isoforms. Treatment of hepatocytes with 5 ng/ml of interleukin-1 $\beta$  mimics the in vivo effects of LPS on CYP4F expression.

© 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.

**Key words:** Inflammation; Leukotriene B<sub>4</sub>  $\omega$ -hydroxylase; Cytochrome P450 4F; Interleukin-1; Lipopolysaccharide; Chlorpromazine metabolism

## 1. Introduction

Leukotrienes are potent eicosanoid lipid mediators derived from phospholipase-released arachidonic acid that are involved in numerous homeostatic biological functions and inflammation. Leukotriene B<sub>4</sub> (LTB<sub>4</sub>), initially identified as an activator of granulocytes [1], exerts its actions by a cell-surface G protein-coupled receptor named BLT1 [2]. LTB<sub>4</sub> initiates and amplifies chemotaxis, cell stimulation and release of granule products/superoxide anions, indicating that it is a key component of inflammatory immune response [3–5]. Very recently, two lines of BLT1-null mice have been generated [6,7]. Neutrophils and macrophages of these mice lacked LTB<sub>4</sub>-induced calcium mobilization and exhibited reduced accumulation of eosinophils, neutrophils and macrophages. The

cytochrome P4504F (CYP4F) subfamily encodes LTB<sub>4</sub>  $\omega$ -hydroxylases that metabolize LTB<sub>4</sub> to biologically less active metabolites [8]. Metabolism of LTB<sub>4</sub> in human polymorphonuclear leukocytes by CYP4F3 to the 20-hydroxy derivative leads to a dramatic loss of both chemotactic and aggregation activity by polymorphonuclear leukocytes [9,10]. Further, 20-OH-LTB<sub>4</sub> is very unstable and is readily metabolized to 20-COOH-LTB<sub>4</sub> [11].

Yeast-expressed human neutrophil LTB<sub>4</sub>  $\omega$ -hydroxylase (CYP4F3) has a  $K_m$  for LTB<sub>4</sub> of 0.64  $\mu$ M [12], consistent with the  $K_m$  for LTB<sub>4</sub> obtained using isolated human neutrophils [9]. The liver form of CYP4F3, which is generated by alternative splicing [13], and another human CYP4F, CYP4F2 [14], are thought to be responsible for removal of LTB<sub>4</sub> from human liver, a major organ for inflammatory mediator inactivation. Apart from LTB<sub>4</sub>, CYP4Fs also metabolize arachidonic acid and its other derivatives such as prostaglandins, lipoxins, and hydroxyeicosatetraenoic acids (HETEs) [12,14–19]. More recently considerable interest with respect to CYP4F function in the kidney has been generated. The ability of arachidonic acid  $\omega$ -hydroxylation, and formation of a potent vasoconstrictor 20-HETE, in human kidney [20] suggests an important role for CYP4Fs in renal functions such as regulation of salt–water balance and arterial blood pressure.

In rats, the CYP4F subfamily consists of four related genes, CYP4F1, CYP4F4, CYP4F5 and CYP4F6 [21,22]. The heterologously expressed rat CYP4Fs show similar substrate specificities to the human isoforms [16,19]. We speculate that the CYP4F family might be able to modulate the extent of inflammation by control of the tissue levels of these important inflammatory mediators. One way to provide support for this hypothesis is to test whether the expression of CYP4Fs changes under inflammatory conditions, since these changes are required to adjust the levels of inflammatory mediators. Administration of bacterial lipopolysaccharide (LPS) has been used extensively as a model of sepsis to study the regulation of P450s during an inflammatory response [23]. BaSO<sub>4</sub>, on the other hand, is a particulate irritant that produces sterile peritonitis and affects the expression of several P450s. Many of the effects of LPS and BaSO<sub>4</sub> on P450 expression are thought to be mediated by cytokines like interleukin-1 (IL-1), IL-6, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interferon- $\gamma$ .

In this study, we first determined the distribution and constitutive expression of CYP4F isoforms in both liver and kidney. Second, we investigated the regulation of hepatic and renal CYP4F gene expressions under inflammatory conditions

\*Corresponding author. Fax: (1)-713-500 0652.

E-mail address: [henry.w.strobel@uth.tmc.edu](mailto:henry.w.strobel@uth.tmc.edu) (H.W. Strobel).

<sup>1</sup> These authors contributed equally to this article.

<sup>2</sup> Present address: Drug Metabolism and Safety, Schering-Plough Research Institute, Kenilworth, NJ 07033, USA.

**Abbreviations:** CYP and P450, cytochrome P450; QRT-PCR, quantitative real time polymerase chain reaction; LTB<sub>4</sub>, leukotriene B<sub>4</sub>; HETE, hydroxyeicosatetraenoic acid; LPS, lipopolysaccharide; IL-1, interleukin-1; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; CPZ, chlorpromazine

using LPS- and BaSO<sub>4</sub>-treated rat models. To delineate whether the effects of LPS were direct and/or indirect, primary cultured rat hepatocytes were challenged with different doses of LPS. The role of IL-1 $\beta$  in regulating CYP4Fs, a predominant pro-inflammatory cytokine released in the liver upon LPS injection, was also tested. Finally, an effort was made to correlate changes in mRNA levels with changes in catalytic activity using non-specific and specific substrates.

## 2. Materials and methods

### 2.1. Animal treatments

Six to eight week old male Fisher 344 rats (Harlan, Indianapolis, IN, USA) were used. The animals were allowed free access to food and water at all times. Chromatographically purified *Escherichia coli* LPS, serotype 0127:B8 (Sigma Chemicals, St. Louis, MO, USA), was dissolved in sterile saline to a final concentration of 1.0 mg/ml by sonication and injected intraperitoneally at a dose of 1.0 mg/kg body weight. BaSO<sub>4</sub> was suspended in phosphate-buffered saline and injected intraperitoneally at a concentration of 5.0 g/kg body weight. Control animals received an equivalent volume of sterile saline. The animals were killed 24 h after treatments by CO<sub>2</sub> asphyxiation. The livers and kidneys were removed for RNA or microsome preparation. These procedures were approved by the Animal Care and Use Committees of both Emory University and the University of Texas Medical School at Houston.

### 2.2. Hepatocyte isolation, culture and treatments

Rat hepatocytes were isolated by in situ collagenase perfusion as described previously [24]. The viability of hepatocyte preparations was 70–85% (trypan blue exclusion) and the yield was 200–400  $\times 10^6$  viable cells/liver. The hepatocytes were plated in Waymouth's medium containing 0.15 mM insulin on 60 mm culture dishes coated with 0.4 ml Matrigel (2.0 mg/ml) at a density of 3.5  $\times 10^6$  cells per dish. Medium was changed to remove the dead cells 4 h after the plating and every 48 h thereafter. Hepatocytes were cultured for 5 days before beginning treatments to allow for recovery of stable expression of P450s. They were treated on day 5 by changing to medium containing specific concentrations of LPS (10 pg/ml–100 ng/ml), IL-1 $\beta$  (5 ng/ml) or vehicle.

### 2.3. RNA and microsome preparations

Total RNA was prepared according to the method of Chomczynski [25]. Microsomes were prepared as described by Saito and Strobel [26]. All procedures were carried out at 4°C. The excised livers were washed and homogenized in buffer A (150 mM KCl, 10 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 50  $\mu$ M vitamin E, pH 7.5). The homogenate was centrifuged at 10000  $\times g$  for 20 min. The pellet was resuspended in buffer A and centrifuged at the same speed. The supernatant fraction was further centrifuged at 105000  $\times g$  for 60 min. The pellet was homogenized in half of the pellet volume of buffer A, and centrifuged under the same conditions. The final pellet was resuspended in buffer B (0.25 M sucrose, 10 mM EDTA, 1 mM PMSF, 250  $\mu$ M vitamin E, and 1 mM dithiothreitol, pH 7.5) and stored at –80°C for further analysis. The microsomal protein concentrations were determined using the bicinchoninic acid procedure [27].

### 2.4. Quantitative real time polymerase chain reaction (QRT-PCR)

All samples were DNase-treated using RQ1 DNase (Promega, Madison, WI, USA). The quality of the isolated RNA was assessed by electrophoresis on 1% agarose gels based on the integrity of 28S and 18S bands after ethidium bromide staining. PCR primers and fluorescent probe sequences for CYP4F isoforms were designed as described previously [28]. Aliquots (200 ng) of total RNA were reverse transcribed in quadruplicate (including an RT blank to account for amplification of contaminating genomic DNA). Amplification was performed using an ABI Prism 7700 (Applied Biosystems, Norwalk, CT, USA) at 95°C for 1 min, followed by 40 cycles of 95°C for 12 s and 60°C for 1 min. Standard curves were generated by plotting Ct versus the log of the amount of amplicon (custom made by IDT, Coralville, IA, USA) for specific 4Fs (500 ag–5 pg), and were used to compare the relative amount of a particular CYP4F mRNA in the samples.

### 2.5. CYP4F immunoblotting

Microsomal protein samples were boiled in Laemmli buffer and resolved on 4–15% Tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Proteins were transferred to nitrocellulose membranes using a semi-dry transfer apparatus. Membranes were blocked overnight with Superblock<sup>®</sup> (Pierce, Rockford, IL, USA) followed by 2 h incubation in 1:250 dilution of polyclonal primary antibody against CYP4F5 (is specific for 4Fs but does not distinguish among the isoforms). Membranes were then washed and incubated at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000 dilution) for 1 h. Immunoreactivity was detected using an HRP chemiluminescence system (Pierce). These experiments were repeated three times each.

### 2.6. Chlorpromazine and LTB<sub>4</sub> metabolism

Metabolism of chlorpromazine (CPZ) and LTB<sub>4</sub> by liver microsomes was determined using high performance liquid chromatography methods as previously described [16,29]. Briefly, the reactions were carried out at 37°C for 20 min in the presence of appropriate buffer, substrate and microsomal concentrations. The reaction products were extracted with 3 ml of ethyl acetate, dried gently under N<sub>2</sub> gas, resububilized in 100  $\mu$ l of mobile phase. These products were passed over a cyano (for CPZ) or C<sub>18</sub> (for LTB<sub>4</sub>) column and measured with a UV detector set at 254 and 270 nm respectively. The quantities of newly formed metabolites were determined from the peak area read from the standard curves prepared for 20-OH-LTB<sub>4</sub> (0.3–5  $\mu$ M), CPZ-sulfoxide and nor-CPZ (1–20  $\mu$ M). The CYP4F activity inhibition was tested by pre-incubating the microsomes with 10  $\mu$ M 17-octadecynoic acid for 10 min at 37°C.

### 2.7. Statistical analysis

Results are expressed as mean  $\pm$  S.E.M. from at least five independent sets of experiments. Comparisons were made using Student's *t*-test and one-way analysis of variance with a post-hoc Tukey's multiple range test. Values of *P* < 0.05 were considered statistically different.

## 3. Results

### 3.1. Constitutive expression and distribution of CYP4Fs in liver and kidney

The expression of each CYP4F isoform in liver and kidney was quantified using QRT-PCR with isoform-specific primers and probes. The amount of each CYP4F isoform in the sample was calculated from the standard curve and normalized to internal control r-cyclophilin. The data are expressed as percentage of total CYP4F expression in liver or in kidney. The results show that CYP4F1 is the dominant isoform in both liver and kidney, accounting for 71.6% and 95.1% of CYP4F expression, respectively (Fig. 1a,b). In liver, CYP4F4 has the second highest expression (18.5%), followed by CYP4F6 (9.8%). The expression of CYP4F5 is only 0.1% of total hepatic CYP4F. In kidney, all other CYP4Fs accounted for only 5% of CYP4F expression, and CYP4F4 was barely detected with this technique.

### 3.2. Regulation of CYP4F expression by LPS and BaSO<sub>4</sub>

The regulation of expression of hepatic and renal CYP4Fs was evaluated at the mRNA level by QRT-PCR. In liver (Fig. 2a), LPS and BaSO<sub>4</sub> did not change the expression of CYP4F1 and 4F6, two major isoforms of CYP4Fs. LPS showed opposite effects on CYP4F4 and 4F5 causing a 50% down-regulation of CYP4F4 and 60% up-regulation of CYP4F5. On the other hand, BaSO<sub>4</sub> led to a 40% increase of CYP4F4 expression. In kidney (Fig. 2b), none of the CYP4F isoforms were affected by LPS treatment, however, BaSO<sub>4</sub> induced the expression of CYP4F1 and 4F6 two- to three-fold over the control levels.

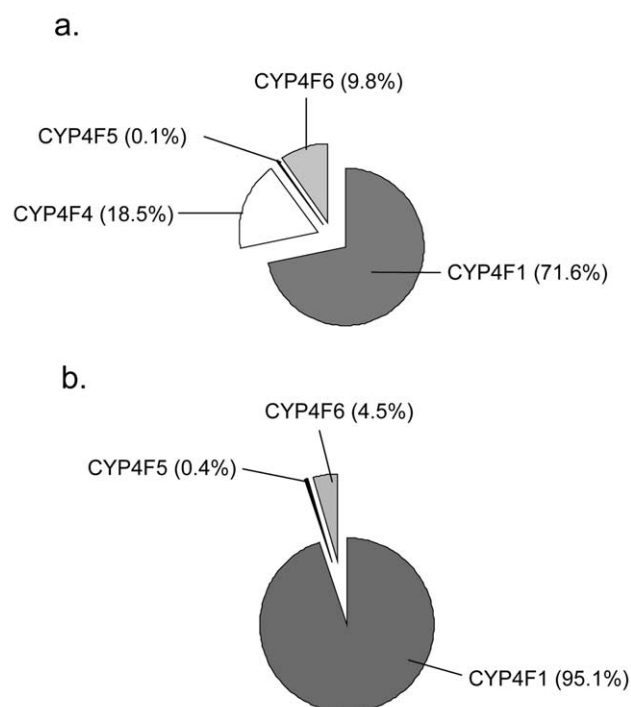


Fig. 1. QRT-PCR analysis of CYP4F isoforms in rat liver and kidney. Total RNA from rat liver or kidney ( $n=5$ ) was prepared and assayed using QRT-PCR as described in Section 2. Each sample was normalized to r-cyclophilin. The pie charts represent the relative distribution of CYP4F isoforms in (a) liver and (b) kidney.

### 3.3. Effect of LPS and BaSO<sub>4</sub> treatment on CPZ metabolism by liver microsomes

The metabolism of CPZ by CYP4F4 and CYP4F5 proteins has been demonstrated by Boehme and Strobel [30], who found nor-CPZ and CPZ-sulfoxide to be the major metabolites. We examined whether LPS or BaSO<sub>4</sub> administration in vivo can affect metabolism of this neuroleptic drug by rat liver microsomes. The major metabolites produced by the microsomes were nor-CPZ and CPZ-sulfoxide, the same as seen when purified CYP4F proteins were used. No hydroxy-CPZ, *N*-oxide-CPZ or nor-CPZ-sulfoxide metabolites were produced (data not shown). The turnover rates were determined from standard curves using pure nor-CPZ and CPZ-sulfoxide. As shown in Table 1, both LPS and BaSO<sub>4</sub> treatment caused a significant decrease in turnover rate for nor-CPZ (58% and 54% respectively), and CPZ-sulfoxide (60% and 68% respectively) formation.

Table 1  
Effect of LPS and BaSO<sub>4</sub> treatment on liver microsome turnover rate for CPZ and LTB<sub>4</sub> metabolism

Treatment	Nor-CPZ	CPZ-sulfoxide	20-OH-LTB <sub>4</sub>
Control	311.1 ± 5.5	3451 ± 313	27.18 ± 0.93
LPS	180.4 ± 4.2*	1855 ± 150*	26.32 ± 2.55
BaSO <sub>4</sub>	188 ± 2.5**	2359 ± 82.7*	34.23 ± 1.63*

CPZ or LTB<sub>4</sub> metabolism was measured in vitro as described in Section 2. Values (pmol formed/min/mg of protein) are the means of determinations on three individual liver microsomal samples. The statistical significance was determined by one-way analysis of variance (\* $P < 0.05$ ; \*\* $P < 0.01$ ).

### 3.4. Effect of LPS and BaSO<sub>4</sub> on CYP4F-mediated LTB<sub>4</sub> metabolism by liver microsomes

LTB<sub>4</sub>, a specific substrate for CYP4Fs, was used as a probe substrate to investigate the effect of LPS and BaSO<sub>4</sub> on CYP4F activity in liver. The  $\omega$ -hydroxylase activity of CYP4Fs was carried out by monitoring the production of 20-OH-LTB<sub>4</sub> as described earlier [16]. The results showed no significant difference between controls and LPS-treated animals while the BaSO<sub>4</sub> treatment resulted in a 30% increase in  $\omega$ -hydroxylase activity (see Table 1). The specificity of the reaction was tested using the CYP4F-specific inhibitor 17-octadecynoic acid, which resulted in up to 53% inhibition of  $\omega$ -hydroxylase activity regardless of the treatment (data not shown). Immunoblot analysis utilizing a polyclonal CYP4F5 antibody revealed a similar degree of CYP4F induction after BaSO<sub>4</sub> treatment while no change was apparent after LPS challenge (see Fig. 3).

### 3.5. Suppression of CYP4F mRNA in hepatocytes treated with LPS

The objective of this experiment was to define the in vitro

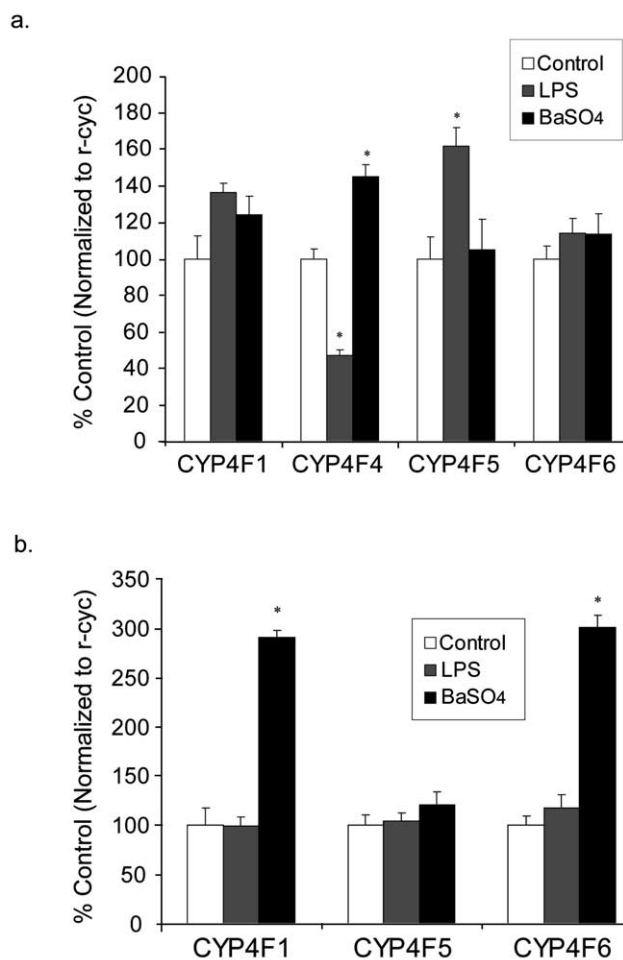


Fig. 2. Effects of LPS and BaSO<sub>4</sub> treatment on hepatic (a) and renal (b) CYP4F isoforms. LPS (1.0 mg/kg) or BaSO<sub>4</sub> (5.0 g/kg) was injected intraperitoneally in rats ( $n=5$ ) as described in Section 2. Total RNA prepared from controls and treated animals was analyzed for individual CYP4F isoform expression by QRT-PCR analysis using r-cyclophilin as an internal standard. \* $P < 0.05$  compared to control.

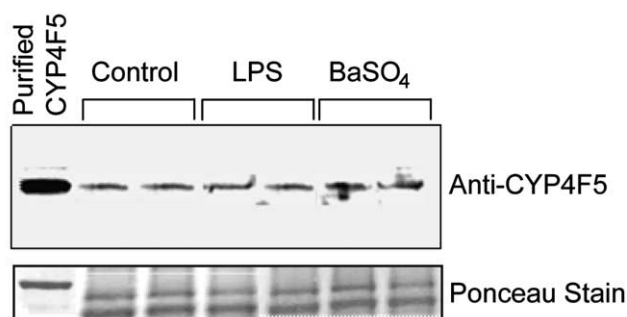


Fig. 3. Immunoblot analysis of CYP4F levels after LPS and BaSO<sub>4</sub> treatments. Liver microsomes were prepared as described in Section 2. 50 µg of microsomal proteins from each sample were separated electrophoretically and then transferred on to nitrocellulose membrane. Membranes were blotted and analyzed using CYP4F5 antibody.

effects of varying doses of LPS on isolated rat hepatocytes and determine whether LPS can act directly on hepatocytes to affect CYP4F expression. Many gene regulation studies have been conducted using primary hepatocyte cultures. It is known that in general P450 expression decreases drastically at the beginning of the culture period, and recovers gradually. The basal expression levels of CYP4F isoforms also decreased after 24 h in culture. However, the expression levels recovered and reached a plateau at day 3–5. Based on these observations, cells were cultured for 5 days before beginning any treatments. As shown in Fig. 4a–d, 24 h treatment of hepa-

tocytes with LPS resulted in a significant decrease in mRNA expression of all CYP4F isoforms. CYP4F1 showed the maximal decrease among the four isoforms with up to 80% suppression observed even at the lowest dose (10 pg/ml) used. A similar degree of suppression in CYP4F4, 4F5 and 4F6 expression was observed at a 100-fold higher dose of LPS. Interestingly, a mild increase in CYP4F4, 4F5 and 4F6 expression was noted at 10 ng when compared to 1 ng of LPS.

### 3.6. IL-1β treatment of hepatocytes mimics the in vivo effects of LPS on CYP4F expression

Treatment of hepatocytes with 5 ng/ml of IL-1 for 24 h resulted in an isoform-specific response by CYP4Fs (Fig. 5). CYP4F1 expression increased by 29% while CYP4F4 expression dropped by 70% when compared to control. In this case, CYP4F5 was the most responsive isoform and showed a 3.5-fold induction upon IL-1 treatment. No significant changes in CYP4F6 levels were observed after IL-1 treatment. Interestingly, these data correlate very well with the hepatic CYP4F response after LPS injection.

## 4. Discussion

Several studies have revealed that the CYP4F subfamily metabolizes leukotriene and prostaglandin products of arachidonic acid cascades, which play a role in inflammation and blood vessel tone regulation. In this study, we used QRT-PCR to analyze CYP4F constitutive expression in liver and kidney, attempting to correlate the distributions with their putative roles in physiological and pathophysiological processes.

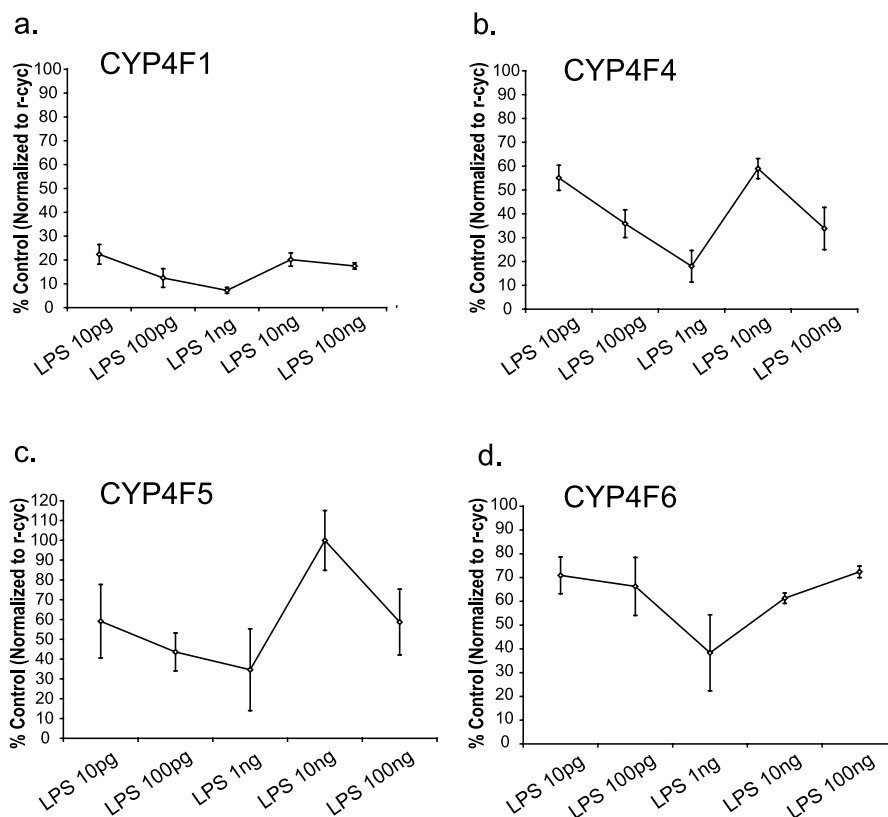


Fig. 4. Concentration-dependent effects of LPS treatment on CYP4F expression in primary hepatocytes. Rat hepatocytes were cultured on Matrigel for 5 days and treated with different concentrations of LPS for 24 h. The expression of CYP4F isoforms was quantitated by QRT-PCR as described earlier. Each data point represents the mean  $\pm$  S.E.M. of five independent culture plates and is expressed as percent of control.

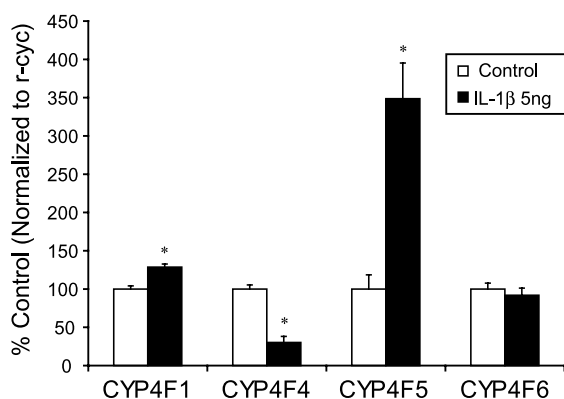


Fig. 5. Effects of IL-1 $\beta$  treatment on CYP4F expression in primary hepatocytes. Hepatocytes were cultured as described in Fig. 3, and treated with 5 ng/ml of IL-1 $\beta$  for 24 h. The expression of CYP4F isoforms was quantitated by QRT-PCR as described earlier. The data represent the mean  $\pm$  S.E.M. of five independent culture plates and are expressed as percent of control. \* $P$  < 0.05 compared to control.

CYP4F1 is the most highly expressed isoform in rat liver and kidney whereas CYP4F5 shows a rather low constitutive expression. Kikuta et al. [17] demonstrated that purified CYP4F1 is quite active as a hydroxylase towards LTB<sub>4</sub>, lipoxins, prostaglandins and HETEs. Such a high expression of CYP4F1 implies it may contribute heavily to the endogenous eicosanoid pool that affects the delicate balance of hepatic and renal physiological functions. This hypothesis is supported by the evidence that besides LTB<sub>4</sub> metabolism, CYP4F-dependent conversion of arachidonic acid to 20-HETE, a potent vasoconstrictor and activator of protein kinase C, may affect important renal functions such as hypertension and water–salt balance [20,31,32]. On the other hand, in a recent report, Bylund et al. [19] have demonstrated that CYP4F5 has a much lower  $K_m$  for LTB<sub>4</sub> (9.7  $\mu$ M for CYP4F5 vs. 26  $\mu$ M for CYP4F6), and is 30 times more efficient than CYP4F6 in metabolizing LTB<sub>4</sub>. The lower constitutive level but higher catalytic efficiency of CYP4F5 compared to other CYP4Fs suggests that CYP4F5 might be an inducible CYP4F isoform and its expression levels during an inflammatory state might be an important determinant of resolution status.

In order to test the role of various CYP4F isoforms in inflammation control, we studied the effects of LPS and BaSO<sub>4</sub>, two separate inflammatory prompts, on CYP4F gene expression. Administration of bacterial LPS has been used extensively as a model of sepsis to study the regulation of P450 genes during an inflammatory response [23]. Similar to LPS, BaSO<sub>4</sub> can cause a systematic inflammatory response but they are different in some aspects. First, LPS injection can directly activate Kupffer cells and cause a high cytokine concentration in the liver as compared with rats which receive BaSO<sub>4</sub> injected intraperitoneally [33]. The cytokine response profiles of the two models are also different. In addition, injection of the particulate irritant is thought to initiate a sterile peritonitis after phagocytosis of these particulates by macrophages [34] or by Kupffer cells after the particulates enter the blood circulation and are transported to the liver [35]. Therefore the response to BaSO<sub>4</sub> is completely macrophage- or Kupffer cell-dependent. In contrast, LPS may directly affect the expressions of some P450s in hepatocytes. Further, LPS administration may induce the expression of nitric oxide syn-

thase, whereas particulate irritants do not [33,36]. Thus, the mechanisms through which LPS and BaSO<sub>4</sub> operate may be different or may share certain mediators such as the cytokines to a greater or lesser degree.

Our results show that CYP4Fs are regulated in a tissue-specific and isoform-specific manner. In liver, CYP4F1 and 4F6 are not affected by either treatment. The closely related CYP4F4 and 4F5 behaved in an opposite fashion upon LPS challenge. The major CYP4Fs in kidney, CYP4F1 and 4F6, only respond to BaSO<sub>4</sub>. Using Northern blot analysis, we previously reported that both CYP4F4 and 4F5 mRNA decrease in liver after LPS and BaSO<sub>4</sub> treatments [24]. The discrepancy in our results from these two studies is most likely a result of lack of specificity and sensitivity of the Northern probes used in the earlier study compared to the present study. The QRT-PCR technique used here is much more sensitive than Northern analysis and provides a specific and better quantitation of individual CYP4F mRNA levels. This discrepancy is clear when we look at relative amounts of CYP4F5 (0.1%) vs. CYP4F4 (18.5%) in liver as determined by QRT-PCR. The CYP4F5 levels in liver are very low in comparison to CYP4F1, 4F4 and 4F6 and therefore even a low degree of cross-hybridization of CYP4F5 probe will adversely affect its quantitation compared to highly abundant other CYP4Fs.

In order to define whether the changes in hepatic CYP4F expression were direct or indirect effects of LPS, cultured rat hepatocytes were subjected to varying concentrations of LPS. All four isoforms displayed a decrease upon LPS challenge with a minor increase seen at a 10 ng dose for CYP4F4 and 4F5. The increase in CYP4F4 and 4F5 levels at higher doses of LPS treatment seems unusual and needs further characterization. However, we speculate that higher doses of LPS may produce additional regulatory mechanisms. The effects of LPS or BaSO<sub>4</sub> on P450 mRNAs are known to be mimicked by administration of interferon inducers and cytokines such as IL-1, IL-6 and TNF- $\alpha$ , indicating that the suppression of CYP gene products in LPS-induced inflammation is likely mediated by inflammatory cytokines [37–39]. Also, cytokines can act directly on hepatocytes to cause changes of CYP gene expression. The mechanisms of this regulation are not known; however, there is evidence that transcriptional events are likely involved. For instance, Chen et al. [37], using a transiently expressed reporter system, showed that CYP2C11 promoter sequences are involved in the suppression of 2C11 by IL-1 and IL-6.

We examined the role of IL-1 $\beta$  in regulating CYP4F expression since in response to LPS it is a major cytokine produced by Kupffer cells and the resident macrophages of the liver [23]. IL-1 $\beta$  selectively induced CYP4F1 and 4F5 expression while CYP4F4 expression was reduced. IL-1 has been shown to mediate its pro-inflammatory effects through nuclear factor- $\kappa$ B (NF- $\kappa$ B), a dimeric transcription factor formed by the hetero- or homodimerization of proteins in the Rel family, including p50 and p60 [40]. NF- $\kappa$ B plays a central role in inflammation through the regulation of genes encoding pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes such as cyclooxygenase-2 and inducible nitric oxide synthase [40]. CYP4F5 genomic characterization revealed there are several NF- $\kappa$ B binding sites in its promoter as well as a 5' flanking region [41]. Whether the other members in the CYP4F subfamily also

contain these sites and whether they are functional remains to be seen.

Interestingly, the results obtained from hepatocyte treatment with IL-1 $\beta$  are quite similar to the in vivo effects of LPS on hepatic CYP4Fs. This suggests that intraperitoneally injected LPS may be causing increased production of cytokines such as IL-1 which directly affect CYP4F expression in liver. On the other hand, dissimilarities in CYP4F regulation by LPS in the in vivo and in vitro experiments might be explained by the fact that the hepatocyte cultures used in this study were essentially devoid of any macrophages or Kupffer cells. Therefore, the chances of cytokine-mediated CYP4F regulation by LPS in this case remain low. In accordance with this view is the study by Milosevic et al. [42] comparing the responses of hepatocytes cocultured with Kupffer cells with those of hepatocytes alone, and showing that suppression of CYP2B1 mRNA by LPS was mediated by TNF- $\alpha$  release from the Kupffer cells. The effects of other pro- and anti-inflammatory cytokines on CYP4F expression are being actively pursued.

To determine whether changes of CYP4F mRNAs in rat liver result in accompanying changes in enzyme activities, we performed CPZ and LTB $_4$  metabolism assays in liver microsomes from different treatment groups. CYP4F4 and 4F5 have been shown to be better catalysts for metabolism of CPZ than CYP1A and 2B [29,30]. The major products of CPZ metabolism by liver microsomes are nor-CPZ and CPZ-sulfoxide, which are the same as the products produced using purified CYP4F isoforms. According to our results, LPS and BaSO $_4$  treatment can suppress demethylation activity to 58% and 60%, respectively, and sulfoxidation activity to 54% and 68%, respectively. In addition to the CYP4F subfamily, activity towards CPZ in the liver microsomes might represent contributions due to the 2C and 2D isoforms as well. Alternatively, when LTB $_4$ , a specific substrate of the CYP4F subfamily, was utilized, the results were completely correlative with mRNA changes. For example, we did not observe any change in LTB $_4$   $\omega$ -hydroxylation after LPS treatment, however, an up-regulation of CYP4F5 and down-regulation of CYP4F4 mRNA was seen. We speculate that the opposite regulation of CYP4F4 and 4F5 may cancel one another leaving no net change in activity. On the other hand, BaSO $_4$  treatment caused a 30% increase in LTB $_4$   $\omega$ -hydroxylation which may be accounted for by the increased CYP4F4 mRNA expression observed upon BaSO $_4$  challenge. Similar results obtained after immunoblot analysis using a polyclonal CYP4F antibody support the above hypothesis and augment the need for isoform-specific antibodies to distinguish each CYP4F form at the protein level. The generation of such antibodies is being actively pursued.

Although inflammation is an integral part of the host defense mechanism against a variety of pathogens as well as tissue injury, it often produces a myriad of unwanted complications. LTB $_4$  is a classical chemotactic agent which acts primarily on the neutrophil to enhance its adherence to endothelium [5]. Adherence of circulating neutrophils to the microvascular endothelium is the initial step in diapedesis, the process by which leukocytes migrate through blood vessels to accumulate at sites of infection or injury [43]. The mechanisms underlying neutrophil–endothelial cell interactions are currently under intense investigation. The proximity of hepatocytes to eicosanoid-producing endothelial cells and Kupffer

cells prompted us to determine the expression and function of LTB $_4$   $\omega$ -hydroxylases during inflammatory conditions. Our findings provide evidence that inflammatory triggers such as LPS and BaSO $_4$  can influence expression of enzymes which are involved in LTB $_4$  breakdown in an isoform- and tissue-specific manner possibly in attempting to re-establish homeostasis. The precise physiological functions of individual CYP4F isoforms remain unclear and deserve further investigation.

**Acknowledgements:** We would like to thank Ms. Sayee Anakk and Ms. Cheri M. Turman for the careful review of the manuscript. This work was supported by Grants MH58297 (H.W.S.) and GM46897 (E.T.M.) from the National Institute of Mental Health and General Medical Sciences, by the DHHW and US Army Grant DAMD-17-98-1-8002 'Disaster Relief and Emergency Medical Service' project and by Harry S. and Isabel Cameron fellowship awarded to A.K.

## References

- [1] Samuelsson, B., Dahlen, S.E., Lindgren, J.A., Rouzer, C.A. and Serhan, C.N. (1987) *Science* 237, 1171–1176.
- [2] Yokomizo, T., Izumi, T., Chang, K., Takuwa, Y. and Shimizu, T. (1997) *Nature* 387, 620–624.
- [3] Borgeat, P. and Naccache, P.H. (1990) *Clin. Biochem.* 23, 459–468.
- [4] Bailie, M.B., Standiford, T.J., Laichalk, L.L., Coffey, M.J., Strieter, R. and Peters-Golden, M. (1996) *J. Immunol.* 157, 5221–5224.
- [5] Ford-Hutchinson, A.W., Bray, M.A., Doig, M.V., Shipley, M.E. and Smith, M.J. (1980) *Nature* 286, 264–265.
- [6] Haribabu, B., Verghese, M.W., Steeber, D.A., Sellars, D.D., Bock, C.B. and Snyderman, R. (2000) *J. Exp. Med.* 192, 433–438.
- [7] Tager, A.M., Dufour, J.H., Goodarzi, K., Bercury, S.D., von Andrian, U.H. and Luster, A.D. (2000) *J. Exp. Med.* 192, 439–446.
- [8] Kikuta, Y., Kusunose, E. and Kusunose, M. (2002) *Prostaglandins Other Lipid Mediat.* 68–69, 345–362.
- [9] Powell, W.S. (1984) *J. Biol. Chem.* 259, 3082–3089.
- [10] Kikuta, Y., Kusunose, E., Endo, K., Yamamoto, S., Sogawa, K., Fujii-Kuriyama, Y. and Kusunose, M. (1993) *J. Biol. Chem.* 268, 9376–9380.
- [11] Yokomizo, T., Izumi, T. and Shimizu, T. (2001) *Arch. Biochem. Biophys.* 385, 231–241.
- [12] Kikuta, Y., Kusunose, E., Sumimoto, H., Mizukami, Y., Takeshige, K., Sakaki, T., Yabusaki, Y. and Kusunose, M. (1998) *Arch. Biochem. Biophys.* 355, 201–205.
- [13] Christmas, P., Ursino, S.R., Fox, J.W. and Soberman, R.J. (1999) *J. Biol. Chem.* 274, 21191–21199.
- [14] Kikuta, Y., Kusunose, E., Kondo, T., Yamamoto, S., Kinoshita, H. and Kusunose, M. (1994) *FEBS Lett.* 348, 70–74.
- [15] Bylund, J., Finnstrom, N. and Oliw, E.H. (1999) *Biochem. Biophys. Res. Commun.* 261, 169–174.
- [16] Kawashima, H., Kusunose, E., Thompson, C.M. and Strobel, H.W. (1997) *Arch. Biochem. Biophys.* 347, 148–154.
- [17] Kikuta, Y., Kusunose, E., Ito, M. and Kusunose, M. (1999) *Arch. Biochem. Biophys.* 369, 193–196.
- [18] Bylund, J., Hidestrand, M., Ingelman-Sundberg, M. and Oliw, E.H. (2000) *J. Biol. Chem.* 275, 21844–21849.
- [19] Bylund, J., Harder, A.G., Maier, K.G., Roman, R.J. and Harder, D.R. (2003) *Arch. Biochem. Biophys.* 412, 34–41.
- [20] Lasker, J.M., Chen, W.B., Wolf, I., Boswick, B.P., Wilson, P.D. and Powell, P.K. (2000) *J. Biol. Chem.* 275, 4118–4126.
- [21] Chen, L. and Hardwick, J.P. (1993) *Arch. Biochem. Biophys.* 300, 18–23.
- [22] Kawashima, H. and Strobel, H.W. (1995) *Biochem. Biophys. Res. Commun.* 217, 1137–1144.
- [23] Morgan, E.T. (1997) *Drug Metab. Rev.* 29, 1129–1188.
- [24] Cui, X. et al. (2003) *Biochim. Biophys. Acta* 1619, 325–331.
- [25] Chomczynski, P. (1993) *Biotechniques* 15, 532–534, 536–537.
- [26] Saito, T. and Strobel, H.W. (1981) *J. Biol. Chem.* 256, 984–988.

- [27] Smith, P.K. et al. (1985) *Anal. Biochem.* 150, 76–85.
- [28] Kalsotra, A., Anakk, S., Boehme, C.L. and Strobel, H.W. (2002) *Drug Metab. Dispos.* 30, 1022–1028.
- [29] Boehme, C.L. and Strobel, H.W. (1998) *J. Chromatogr. B Biomed. Sci. Appl.* 718, 259–266.
- [30] Boehme, C.L. and Strobel, H.W. (2001) *Neurotoxic. Res.* 3, 329–337.
- [31] Stec, D.E., Flasch, A., Roman, R.J. and White, J.A. (2003) *Am. J. Physiol. Renal Physiol.* 284, F95–F102.
- [32] Christmas, P. et al. (2001) *J. Biol. Chem.* 276, 38166–38172.
- [33] Sewer, M.B., Koop, D.R. and Morgan, E.T. (1997) *J. Pharmacol. Exp. Ther.* 280, 1445–1454.
- [34] Ufkes, J.G., Ottenhof, M., van Rooij, H.H. and van Gool, J. (1988) *Br. J. Exp. Pathol.* 69, 457–464.
- [35] Peterson, T.C. and Renton, K.W. (1986) *Biochem. Pharmacol.* 35, 1491–1497.
- [36] Geller, D.A. et al. (1994) *Arch. Surg.* 129, 165–171.
- [37] Chen, J.Q., Strom, A., Gustafsson, J.A. and Morgan, E.T. (1995) *Mol. Pharmacol.* 47, 940–947.
- [38] Morgan, E.T. (1991) *Biochem. Pharmacol.* 42, 51–57.
- [39] Morgan, E.T., Thomas, K.B., Swanson, R., Vales, T., Hwang, J. and Wright, K. (1994) *Biochim. Biophys. Acta* 1219, 475–483.
- [40] Baldwin Jr., A.S. (1996) *Annu. Rev. Immunol.* 14, 649–683.
- [41] Cui, X. and Strobel, H. (2002) *Gene* 297, 179.
- [42] Milosevic, N., Schawald, H. and Maier, P. (1999) *Eur. J. Pharmacol.* 368, 75–87.
- [43] Springer, T.A. (1994) *Cell* 76, 301–314.