Down-Regulation of Hepatic Cytochrome P450 Enzymes in Rats with Trinitrobenzene Sulfonic Acid-Induced Colitis

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ABSTRACT:

Hepatic cytochrome P450 (P450) enzymes are down-regulated during inflammation. In this study, an animal model of inflammatory bowel disease was subject to characterization of hepatic P450 expression under inflammatory conditions. Rats were treated intracolonically with 100 mg/kg trinitrobenzene sulfonic acid (TNBS) dissolved in 30% ethanol, and homogenates of colonic mucosa and hepatic microsomes of the rats were prepared. The colitis was accompanied by appearance of higher levels of portal endotoxin, interleukin-6, and nitric oxide metabolites and decreases in contents and activities for hepatic CYP3A2, CYP2C11, and, to a lesser extent, CYP1A2 and CYP2E1. Nimesulide, a preferential COX-2 inhibitor, protected rats with TNBS-induced colitis (TNBS-colitis) against the down-regulation of hepatic CYP3A2. Polymyxin B, which neutralizes endotoxin, curcumin, which has anti-inflammatory properties, and gadolinium chloride, which inactivates macrophages, attenuated the down-regulation of CYP3A2. Similar effects were observed in other P450s such as CYP2C11, but the agents were less effective in attenuating the down-regulation. Our data suggest that endogenous substances leaked from damaged colon in the rats with TNBS-colitis activate Kupffer cells, leading to down-regulation of hepatic P450s with differential susceptibility to the inflammatory stimuli. The colitis model, instead of exogenous administration of lipopolysaccharide or cytokines, could be applied to the study on mechanisms for altered hepatic P450 expression and other liver functions under mild inflammatory conditions.

Cytochrome P450 (P450) enzymes in the liver are markedly down-regulated during inflammation. Exogenous administration of lipopolysaccharide (LPS) is widely used as an inflammation model, and various findings have been obtained from the LPS models, which led to advances in understanding transcriptional regulation of hepatic P450s (Morgan, 1997; Renton, 2001). Because of its various dosage regimens, all of the LPS models may not faithfully reproduce exposure of the liver to endogenous mediators derived from sites of inflammation. To characterize the down-regulation of hepatic P450 enzymes during mild inflammation, we attempt to provide here a model of inflammatory bowel disease as an alternative to the LPS models. We previously developed chemically induced ileitis and colitis models by intraperitoneal administration of a nonsteroidal anti-inflammatory drug, indomethacin, and treatment of rats with dextran sulfate sodium in drinking water, respectively, resulting in down-regulation of hepatic P450 enzymes (Masubuchi et al., 2002; Masubuchi and Horie, 2004). However, because of their chemical properties and routes of administration, it is also possible that the treatments involve direct influence of the chemicals on liver P450s to some extent.

Intrarectal administration of a hapten, trinitrobenzene sulfonic acid (TNBS), induces colonic inflammation. It is widely used as a human inflammatory bowel disease model and is demonstrated to be T-cell-dependent (Morris et al., 1989; Neurath et al., 1995; Dohi et al., 2000). Several studies investigated liver function along with portal release of endotoxin in the rats with TNBS-induced colitis (TNBS-colitis). One observed low-grade portal and systemic endotoxemia without hepatobiliary alteration (Brand et al., 1994). On the other hand, an increase in hepatocyte tight-junction permeability and impaired biliary function observed in the rats with TNBS-colitis suggest association of the colitis with hepatobiliary complications (Lora et al., 1997; Kawaguchi et al., 2000). These results imply that hepatic P450 enzymes, which are highly sensitive to inflammatory stimuli as described above (Morgan, 1997; Renton, 2001), are down-regulated and/or functionally disordered. In fact, the liver perfusion study, although it did not directly determine P450 content or P450-dependent enzyme activity, suggested disturbance of the P450 enzyme function in the rats with TNBS-colitis (Weidenbach et al., 2000).

In the present study, rats were treated intracolonically with 100 mg/kg TNBS dissolved in 30% ethanol to develop colitis, and we demonstrate here that the colitis accompanies down-regulation of...
hepatic P450 enzymes in the isoform-dependent manner. In addition, attempts have been made to protect against the down-regulation of hepatic P450s by using chemical agents to assess the role of inflammatory mediators in the liver effects.

Materials and Methods

Chemicals. TNBS was purchased from Fluka (Milwaukee, WI); 4-nitropheno- phenol, 4-nitrocatechol, and propranolol hydrochloride were from the Wako Pure Chemical (Osaka, Japan); testosterone, phenacetin, and 4-acetamidophenol were from Sigma-Aldrich (St. Louis, MO); 2α-, 6β-, and 16α-hydroxysterosterones were from Steraloids Inc. (Wilton, NH). 4-Hydroxypropranolol hydrochloride was donated from Sumitomo Chemical (Osaka, Japan). Glucose 6-phosphate (G-6-P), glucose 6-phosphate dehydrogenase (G-6-PDH), and NADPH were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). All other chemicals and solvents used were of analytical grade.

Animals. Male Wistar rats (2 months old) were obtained from Takasugi Experimental Animals (Saitama, Japan). The animals were housed in an air-conditioned room (25°C) under a 12-h light/dark cycle for 1 week before use. Food [commercially available pellet (Oriental Yeast Co., Ltd.)] and water were given ad libitum. All animal experiments were performed according to the criteria for humane care as outlined in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996).

Experimental Design. Colitis was induced using the method described by Morris et al. (1989). Rats were treated intracolonically with 100 mg/kg TNBS dissolved in 30% ethanol. Control rats were treated with 30% ethanol alone. The rats were killed 1, 2, 3, or 7 days after the administration along with the untreated rats. In experiments to examine the effects of the chemical agent, some rats were given polymyxin B (72 mg/kg in drinking water) for 4 days from 1 day before TNBS; curcumin (300 mg/kg, p.o.) for 5 days from 2 days before TNBS; nimesulide (10 mg/kg, s.c.) with TNBS; or gondolinium chloride (7.5 mg/kg, i.v.) with TNBS. In these experiments, the rats were killed 3 days after the TNBS treatment. Blood for the assay of alanine aminotransferase (ALT) was sampled from the carotid artery and that for the assay of the portal endotoxin, cytokines, and nitric oxide (NO) was sampled from the cannulated portal vein under anesthesia with diethyl ether. The blood was allowed to coagulate, and the samples were then centrifuged to obtain serum. Colon mucosa was harvested with a glass slide and was homogenated.

Assessment of Mucosal Damage and Biochemical Analysis. Severity of macroscopically observed hemorrhage colitis was evaluated by damaged area and myeloperoxidase (MPO) activity. The damaged area in colonic mucosa was assessed with digital photographs from colon samples, and the values were calculated according to the method of Kankuri et al. (2001). MPO of colonic mucosa was measured as a marker of tissue granulocyte infiltration according to the reported method (Schierwagen et al., 1990) with modifications. Colonic samples were placed in 0.5% hexadecyltrimethylammonium bromide solution to lyse the cells, and the samples were then centrifuged to obtain serum. The supernatant was harvested with a glass slide and was homogenated.

Assay of Liver Microsomal P450 Proteins. Liver microsomal fractions were prepared according to the method of Omura and Sato (1964) for each individual animal separately. Protein concentrations were assayed by the method of Lowry et al. (1951). P450 isozyme content was determined by immunoblot analysis as previously reported (Masubuchi et al., 2002) with modifications. Microsomal proteins (0.5–2.0 µg) were separated by SDS-polyacrylamide gel electrophoresis with an 8% polyacrylamide gel. The proteins on the gel were transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) followed by treatment of the membrane with each polyclonal antibody against CYP3A2, CYP2C11, CYP1A1, and CYP2E1 (Daiichi Pure Chemical Co., Ltd., Tokyo, Japan), which were diluted 1:2000 for use. According to the manufacturer, anti-CYP3A2 antibodies do not cross-react with other major P450s expressed in rats. On the other hand, anti-CYP2C11 antibodies recognize CYP2C6 with lesser reactivity than CYP2C11. Anti-CYP1A1 antibodies recognize CYP1A1 and CYP1A2, which were separated by SDS-polyacrylamide gel electrophoresis. Anti-CYP2E1 antibodies recognize CYP1A and CYP2C enzymes, but the reactivities are much lower than those with the antigen. The immunoblots were developed with the enhanced chemiluminescence detection method with reagents from Amersham Pharmacia Biotech (Uppsala, Sweden) according to the manufacturer’s instructions. The intensities of the stained bands were measured by laser densitometry, and the relative intensities to those of control microsomes were calculated as the relative P450 isozyme contents.

Assay of Liver Microsomal Monooxygenase Activities. Testosterone 2α-, 6β-, and 16α-hydroxylation, phenacetin O-deethylation, 4-nitropheno- hydrolase, and propranolol 4-hydroxylation activities were determined according to the reported high-performance liquid chromatography (HPLC) methods (Masubuchi and Horie, 2003) with minor modifications. A 1-ml incubation mixture for a regular assay procedure contained 0.5 mg/ml liver microsomes, 10 mM G-6-P, 2 units/ml G-6-PDH, 5–10 mM MgCl2; and each substrate in 0.15 M potassium phosphate buffer (pH 7.4), except for phenacetin O-deethylation, which was assayed in a 1-ml mixture with 0.15 M Tris-HCl buffer (pH 7.4), and p-nitrophenol 4-hydroxylation, which was assayed in a 0.5-ml mixture with potassium phosphate buffer (pH 6.8). Substrate concentrations used were: testosterone, 50 µM; phenacetin, 10 µM; 4-nitropheno- phol, 50 µM; and propranolol, 20 µM. After temperature equilibration (37°C, 5 min), the reaction was started by adding NADPH (final 0.5 mM), and the incubation was performed for optimal periods: testosterone, 2.5 min; phenacetin, 10 min; 4-nitropheno- phol, 10 min; propranolol, 2 min. Thus, all of the assays were performed under linear conditions of metabolite formation with regard to incubation time. The metabolites were extracted into ethyl acetate, the organic layer was evaporated to dryness, and the residue was dissolved in 0.1 ml of a mobile phase for the HPLC. The HPLC conditions were the same as the reported method (Masubuchi and Horie, 2003) except for a reversed-phase column (Inertsil ODS 3; GL Sciences Ltd., Tokyo, Japan) commonly applied for the assays in this study.

Results

TNBS-Colitis without Liver Injury. Male Wistar rats treated intracolonically with 100 mg/kg TNBS in 30% ethanol developed ulcerative colitis-like symptoms. Hemorrhage colitis was macroscopically observed (Fig. 1), and mucosal injury was demonstrated by a marked increase in MPO activity of colonic mucosa (Fig. 2B). The damaged area (Fig. 2A) and MPO activities (Fig. 2B) reached maximal values 2 days after the TNBS treatment, whereas the former but not the latter returned to near baseline by 7 days after the treatment. Endotoxin was detected in the portal blood of the rats treated with TNBS, which was higher than that in control rats (Table 1), indicating

Fig. 1. Hemorrhagic colitis induced by TNBS. Rats were treated intracolonically with 100 mg/kg TNBS dissolved in 30% ethanol or 30% ethanol alone (control), and colon samples were harvested 3 days after dosing.
that TNBS-induced colitis resulted in portal endotoxemia. Similar increases in portal concentration were observed for IL-6 (Table 1) and NO metabolites (Fig. 2D) but not in TNF-$\alpha$ (Table 1). Despite the elevated portal endotoxin, there was no sign of liver injury in the rats with TNBS-colitis as judged by serum ALT leakage (Fig. 2C).

**Down-Regulation of Hepatic P450 Enzymes in Rats with TNBS-Colitis.** Protein contents of P450 enzymes and the following liver microsomal oxidation activities were measured as indexes of specific P450 enzymes expressed in normal male rat liver (Reinke and Moyer, 1985; Imaoka et al., 1988; Sesardic et al., 1990; Masubuchi et al., 1993): testosterone 6$\beta$-hydroxylation, CYP3A2; testosterone 16$\alpha$-hydroxylation, CYP2C11; phenacetin O-deethylation, CYP1A2; 4-nitrophenol hydroxylation, CYP2E1; propranolol 4-hydroxylation, CYP2D1/2. CYP3A2 content and activity began to decline 1 day after the TNBS treatment, continued to low levels for 3 days, and returned to near control level 7 days after the treatment (Fig. 3, A and B).

**TABLE 1**

<table>
<thead>
<tr>
<th>Endotoxin</th>
<th>TNF-$\alpha$</th>
<th>IL-6</th>
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<tbody>
<tr>
<td>pg/ml</td>
<td>pg/ml</td>
<td>pg/ml</td>
</tr>
<tr>
<td>Control</td>
<td>5.9 ± 0.9</td>
<td>24.1 ± 1.5</td>
</tr>
<tr>
<td>TNBS</td>
<td>34.8 ± 11.7*</td>
<td>30.2 ± 9.3</td>
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*P < 0.05 vs. control.

Although the immunoblotting performed in the present study may not discriminate CYP3A2 and CYP3A23 proteins, it is considered that protein detected by the anti-CYP3A2 antibody is predominantly CYP3A2, because CYP3A2 is a major P450 isoform in untreated male rats, whereas CYP3A23 is an inducible isoform expressed at minimal constitutive levels (Agrawal and Shapiro, 2003). Similar results were obtained for CYP2C11, while decrease in the activity was more pronounced than the protein content (Fig. 3, C and D). Anti-CYP2C11 antibodies recognize CYP2C6, another major CYP2C enzyme, with lesser reactivity than CYP2C11, and it may be one of the reasons for a moderate decrease in CYP2C11 content. The decreases in CYP1A2 (Fig. 3, E and F) and CYP2E1 (Fig. 3, G and H) were observed only for activities and to lesser extents than those of CYP3A2 and CYP2C11. No clear change was observed for propranolol 4-hydroxylation activity (data not shown).

**Chemical Protection of Rats against the Down-Regulation of Hepatic P450 Enzymes.** To examine roles of enteric bacteria and their cell wall components in the down-regulation of CYP3A2 and other P450 enzymes, an antibiotic polymyxin B, which binds to and neutralizes endotoxin, was coadministered with TNBS. To examine roles of inflammatory mediators including cytokines and prostaglandins, effects of curcumin, which has anti-inflammatory properties, and nimesulide, a preferential cyclooxygenase-2 (COX-2) inhibitor, were tested. To examine roles of Kupffer cells, gadolinium chloride, which inactivates macrophages, was subjected to the experiments. Nimesulide completely protected the rats with TNBS-colitis against the down-regulation of hepatic CYP3A2 (Fig. 4A). Polymyxin B, curcumin, and gadolinium chloride attenuated the decrease in CYP3A2,
FIG. 3. Down-regulation of hepatic P450 enzymes in the rats with TNBS-colitis. Rats were treated intracolonically with 100 mg/kg TNBS dissolved in 30% ethanol (●) or 30% ethanol alone (○). Livers of the rats were obtained at various time points, and microsomal P450 contents (left) and activities (right) were determined as follows. A, CYP3A2 content; B, CYP3A2 activity (testosterone 6β-hydroxylation); C, CYP2C11 content; D, CYP2C11 activity (testosterone 16α-hydroxylation); E, CYP1A2 content; F, CYP1A2 activity (phenacetin O-deethylation); G, CYP2E1 content; H, CYP2E1 activity (4-nitrophenol hydroxylation). Results are means ± S.E. of 4 to 8 rats. *p < 0.05 versus control. Mean control activities are: B, 1.64; D, 1.01; F, 0.198; and H, 1.34 nmol/min/mg protein.
but the CYP3A2 activities in the pretreated rats were still lower than controls (Fig. 4A). The treatment with these drugs did not protect the rats against the induction of TNBS colitis, which was evaluated macroscopically (data not shown). These treatments were less effective on the down-regulation of hepatic CYP2C11 (Fig. 4B). The decreases in CYP1A2 and CYP2E1, which were less susceptible to the TNBS colitis as described above, were also attenuated by the treatment with curcumin, nimesulide, and gadolinium chloride, whereas polymyxin B was commonly less effective than those of the anti-inflammatory agents (Fig. 4, C and D).

**Discussion**

As previously reported (Morris et al., 1989), the present study revealed that treatment of rats intracolonically with 100 mg/kg TNBS dissolved in 30% ethanol developed ulcerative colitis-like symptoms. Macroscopically observed hemorrhage colitis was observed 1 day after the treatment of TNBS and was recovered at 7 days after the treatment, which was slightly different from other parameters, MPO activities in colonic mucosa, and portal NO metabolites, which were probably released from injured colon. It was also observed that the TNBS treatment increased portal endotoxin, which has been reported in the previous studies (Brand et al., 1994; Kawaguchi et al., 2000; Weidenbach et al., 2000), suggesting development of mild endotoxemia in this colitis model. There was no sign of liver injury in the rats with TNBS-colitis, which may be simply due to their endotoxin concentrations, which were not high enough to cause hepatocyte injury. On the other hand, higher levels of portal IL-6 but not TNF-α were observed, as was reported previously (Neill et al., 1995). Therefore, it is also possible that a component highly leaked in the portal vein such as IL-6 acts as a protective factor and prevents rats from endotoxin-induced liver injury, since a protective effect of IL-6 against LPS-induced liver injury has been reported (Inoue et al., 2005). Although a change in serum ALT was negligible, it cannot be ruled out that there are more subtle changes in liver morphology/biochemistry reflective of toxicity, and it is implicated in the decreases in hepatic P450 enzymes described below.

The present study demonstrated down-regulation of hepatic CYP3A2 and CYP2C11 and, to a lesser extent, CYP1A2 and CYP2E1 during the development of TNBS-induced colitis. P450 function in the liver was reported to be disturbed in the rats with TNBS-induced colitis, which was estimated from monoethylglycinexylidide (MEGX) appearance in the perfusate after dosing lidocaine in the perfused liver system (Weidenbach et al., 2000). It was demonstrated that multiple P450 enzymes had an ability to metabolize lidocaine into MEGX, and male-specific P450 enzymes, that is, CYP2C11 and CYP3A2, were largely responsible for it in male rat liver microsomes (Imaoka et al., 1990; Masubuchi et al., 1991). Therefore, the down-regulation of CYP3A2 and CYP2C11, which was shown in the present study for the first time, might contribute to proposed P450 disturbance in the perfused rat liver. However, effects of steps other than MEGX formation, such as hepatic uptake of lidocaine and degradation of MEGX on the rate of appearance of MEGX, cannot be excluded in liver perfusion and in vivo studies.

The down-regulation of hepatic P450 enzymes without sign of liver injury in the rats with TNBS-colitis can be accounted for by high sensitivity of the P450 enzymes to inflammatory responses. Furthermore, differences in susceptibilities between P450 enzymes were also observed: high sensitivity of CYP3A2 and CYP2C11 followed by
CYP1A2 and CYP2E1, whose changes were observed only for enzyme activities, and no change in CYP2D activity. It is postulated that inflammatory mediators derived from injured colon impair CYP1A2 and CYP2E1 activities rather than down-regulation. For example, nitric oxide, one of the possible inflammatory mediators, interacts with and impairs P450 function without changing P450 contents measured by the immunoblotting (Minamiyama et al., 1997). Lack of effects on the CYP2D enzyme also has been obtained from the rats with LPS injection (Shimamoto et al., 1998) and the rats with dextran sulfate sodium-induced colitis (Masubuchi and Horie, 2004). It has been reported that an intraperitoneal dose of LPS causes down-regulation of CYP1A2 and CYP2E1, but it required a relatively high dose (Renton and Nicholson, 2000). It is evident that CYP1A2 and CYP2E1 are also less susceptible to inflammatory stimuli than CYP3A2 and CYP2C11; in other words, more severe inflammation may lead to nonspecific down-regulation of hepatic P450 enzymes. A recent study reported that TNBS impaired intestinal expression of CYP1A1 and CYP1B1 (Coste et al., 2007). It is possible that such direct action of TNBS also contributes to the effect on hepatic P450s that are susceptible to TNBS, such as CYP2C11.

We attempted to protect rats with TNBS-colitis against the down-regulation of hepatic P450 enzymes by chemical agents. There were some differences between P450 isoforms as to extents of the protection, and the results are summarized as follows with those of CYP3A2 (Fig. 4A) as representative. Incomplete protection was obtained against the down-regulation of hepatic CYP3A2 by polymyxin B, which neutralizes endotoxin, although higher portal endotoxin was detected in the rats with TNBS-colitis than in control rats. It is simply possible that the endotoxin-independent pathway contributes to the down-regulation of hepatic CYP3A2. A previous study, which showed an increased portal endotoxin in the TNBS-colitis rats to the same extent as the present study, proposed that mild portal endotoxemia may not be enough to lead to liver dysfunction (Weidenbach et al., 2000). Curcumin, which possesses anti-inflammatory properties, protected rats against the down-regulation of hepatic CYP3A2 and, to a lesser extent, CYP2E1 and CYP2C11. It was reported that curcumin blocked LPS-induced suppression of CYP3A2, partially blocked the suppression of CYP2E1, but did not block those of CYP2C11 (Cheng et al., 2003). Thus, the relative susceptibility of CYP3A2 to curcumin-induced protection is similar in the LPS model and the TNBS-induced colitis model. Although curcumin is known to inhibit NF-κB activation, it may not be involved in the blocking because induction of known NF-κB-regulated genes was not attenuated (Cheng et al., 2003). Therefore, NF-κB-independent mechanisms on colitis-induced down-regulation as well as P450 induction in TNBS-induced damage should be elucidated in the future study. Nimesulide, a preferential COX-2 inhibitor, also protected rats with TNBS-colitis from the down-regulation of hepatic CYP3A2, suggesting involvement of COX-2 products, such as prostaglandin E2. COX-2 is shown to exert proinflammatory effects in the liver and be associated with cytokine production (Yu et al., 2006), which may down-regulate hepatic P450 enzymes. It is unclear why nimesulide effectively blocks the down-regulation, but it is possible that a COX-2-dependent mediator(s) involves hypothetical multiple pathways, such as, for instance, endotoxin-dependent and -independent pathways. The attenuation of the down-regulation of hepatic CYP3A2 by gadolinium chloride, which inactivates macrophages, suggests an important role of Kupffer cells in generation and transduction of inflammatory signals to hepatocytes. The involvement of Kupffer cells in the down-regulation of hepatic P450 enzymes has been also observed in the rat model of indomethacin-induced intestinal injury (Masubuchi et al., 2002).

As described above, hepatic P450 enzymes are down-regulated under inflammatory conditions, which proceed partly via endotoxin-independent pathways. Therefore, LPS models may not fully comprehend pathways for down-regulation of hepatic P450 enzymes under inflammatory conditions. Conversely, a high-dose LPS may suppress hepatic P450s, not only by physiologically relevant inflammation, but by nonspecific effects of LPS. In summary, the TNBS-colitis did not elicit liver injury but caused down-regulation of hepatic P450 enzymes. Multiple inflammatory mediators, endotoxin, cytokines, and prostaglandins are responsible for the down-regulation of hepatic P450 enzymes under inflammatory conditions. The colitis model is thus useful not only for studying pathogenesis of ulcerative colitis but also as a tool to alter the liver function by the endogenous inflammatory mediators without injection of LPS.

References

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