Metabolic Activation and Inflammation Reactions Involved in Carbamazepine-Induced Liver Injury

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Received May 17, 2012; accepted July 3, 2012

Drug-induced liver injury is a major safety concern in drug development and clinical pharmacotherapy; however, advances in the understanding of the mechanisms of drug-induced liver injury are hampered by the lack of animal models. Carbamazepine (CBZ) is a widely used antiepileptic agent. Although the drug is generally well tolerated, only a limited number of patients prescribed CBZ develop severe hepatitis. In the present study, we developed a mouse model of CBZ-induced liver injury and elucidated the mechanisms accounting for the hepatotoxicity of CBZ. Male BALB/c mice were orally administered CBZ for 5 days. The plasma levels of alanine aminotransferase and aspartate aminotransferase were prominently increased, and severe liver damage was observed via histological evaluation. The analysis of the plasma concentration of CBZ and its metabolites demonstrated that 3-hydroxy CBZ may be relevant in CBZ-induced liver injury. The hepatic glutathione levels were significantly decreased, and oxidative stress markers were significantly altered. Mechanistic investigations found that hepatic mRNA levels of toll-like receptor 4, receptor for advanced glycation end products, and their ligands were significantly increased. Moreover, the plasma concentrations of proinflammatory cytokines were also increased. Prostaglandin E2 administration ameliorated the hepatic injury caused by CBZ. In conclusion, metabolic activation followed by the stimulation of immune responses was demonstrated to be involved in CBZ-induced liver injury in mice.

Key Words: carbamazepine; IL-17; hepatotoxicity; metabolism; mouse model.

Adverse drug reactions are a clinical concern and cause attrition in drug development, with hepatotoxicity being a major contributor. It is thought that many idiosyncratic drug reactions result from the production of reactive metabolites by cytochrome P450 (CYP) enzyme systems. The reactive metabolites may lead to hepatic injuries either by direct or by immune-related mechanisms (Park et al., 2000).

Carbamazepine (CBZ) is a widely used antiepileptic agent. Although the drug is generally well tolerated, only a limited number of patients prescribed CBZ develop severe, potentially life-threatening idiosyncratic reactions such as agranulocytosis, hepatitis, and Stevens-Johnson syndrome (Björnsson and Olsson, 2005; Kaufman and Shapiro, 2000; Pallock, 1987). The reasons why only few individuals are affected are poorly understood. Serious CBZ-associated hepatotoxicity assumes the following two forms: (1) a hypersensitive reaction in the form of granulomatosus hepatitis that presents with fever and abnormal liver functions and (2) an acute hepatitis and hepatocellular necrosis with fever and inflammation (Björnsson, 2008; Björnsson and Olsson, 2005). In addition, the presence of a specific autoantibody directed against a human liver microsomal protein in a patient who had severe hepatotoxicity with CBZ has been reported (Pirmohamed et al., 1992a). Based on these reports, the liver injury associated with CBZ is thought to have an immunological basis. In contrast, another antiepileptic drug, oxcarbazepine (OXC), is not likely to be associated with idiosyncratic hepatotoxicity in humans, and there have been only a few case reports showing mild or transitory liver injury (Ahmed and Siddiqi, 2006; Björnsson, 2008).

CBZ is extensively metabolized in humans to over 30 metabolites, and the most important pathway involves the formation of the stable CBZ-10,11-epoxide followed by hydroxylation to CBZ-10,11-trans-dihydroxy (diOH) (Lertratanangkoon and Horning, 1982; Lu and Uetrecht, 2008). Chemically reactive metabolites are also generated as shown in Figure 1. Numerous studies have assayed CBZ-induced cytotoxicity by reactive metabolites in vitro, and they have suggested that CBZ 2,3-epoxide and 3-hydroxy (OH) CBZ might play a role in CBZ-induced idiosyncratic drug reactions via covalent binding to the protein or the production of reactive oxygen species (ROS) (Lu and Uetrecht, 2008; Pirmohamed et al., 1992b), and glutathione (GSH) and microsomal epoxide hydrolase are involved in detoxification (Pirmohamed et al., 1992b). However, there are no reports of CBZ-induced hepatotoxicity in an in vivo animal model.

It is well known that ROS is involved in drug-induced liver injury via mitochondrial dysfunction or hepatocyte necrosis (Jaeschke et al., 2002), and it was recently reported that ROS increased the expression of the toll-like receptor (TLR) and the
receptor for advanced glycation end products (RAGE), as well as their ligands, such as S100 protein and high-mobility group box 1 (HMGB1) (Yao and Brownlee, 2010). The activation of TLR or RAGE results in the induction of inflammatory cytokines and chemokines (Lotze et al., 2007). Cytokines and chemokines, followed by inflammation or the infiltration of lymphocytes to hepatocytes, are involved in immune-mediated hepatotoxicity, and they are predominantly secreted from immune cells such as T lymphocytes and macrophages (Kita et al., 2001; Oo and Adams, 2009). Cytokines are generated by several transcriptional factors: T-box expressed in T cells (T-bet) induces the secretion of interferon (IFN)-γ and interleukin (IL)–12; GATA-binding domain-3 (GATA-3) induces IL-4, IL-5, and IL-13 production; and retinoid-related orphan receptor (ROR)-γt promotes the production of IL-6 and IL-23, which leads to an increase in IL-17 generation (Kidd, 2003; Langrish et al., 2005; Steinman, 2007).

IL-17, a T helper (Th) 17-type cytokine, acts as a potent inflammatory cytokine, and it is detected in sera and target tissues of patients with various immune-related diseases, including rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, and asthma (Kidd, 2003; Steinman, 2007). Consistent with those observations, it has been suggested that IL-17 is involved in the pathogenesis of immune-mediated hepatotoxicities in mice, such as halothane- or α-naphthylisothiocyanate-induced hepatotoxicity (Kobayashi et al., 2009, 2010).

At present, the widely studied model of drug-induced liver injury is the acetaminophen (APAP)-induced liver injury model, which commonly involves mice as the model organism. Although important information on the mechanisms of drug-induced acute inflammatory injury has been generated from this model, APAP hepatotoxicity does not encompass all possible mechanistic features of drug-induced liver injury. Therefore, it is critical to establish several animal models of drug-induced liver injury. This work is the first study to establish a mouse model of CBZ-induced hepatotoxicity. Our data suggest that metabolic activation and inflammation reactions are involved in CBZ-induced liver injury, and this is an appropriate animal model for the study of the severe hepatotoxicity induced by CBZ.

MATERIALS AND METHODS

Materials. CBZ was purchased from Wako Pure Chemical Industries (Osaka, Japan). OXC was from LKT Laboratories (St Paul, Minnesota), and 2-OH CBZ and 3-OH CBZ were from Toronto Research Chemicals (Toronto, Canada). CBZ-10,11-epoxide and trans-10,11-dioH CBZ were kindly provided by Novartis Pharma Inc. (Basel, Switzerland). RNAiso was from Nippon Gene (Tokyo, Japan). Fuji DRI-CHEM slides of GPT/ALT-PIII and GOT/AST-PIII used to measure alanine aminotransferase (ALT) and aspartate aminotransferase (AST), respectively, were purchased from Fujifilm (Tokyo, Japan). ReverTra Ace was from Toyobo (Tokyo, Japan). Random hexamers and SYBR Premix Ex Taq were from Takara (Osaka, Japan). Primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Eritoran was kindly provided by Eisai Co. (Tokyo, Japan). Prostaglandin E1 (PGE1) was purchased from Nippon Chemiphar (Tokyo, Japan). The monoclonal anti-mouse IL-17 antibody, monoclonal anti-mouse/rat RAGE antibody, and monoclonal rat IgG2a isotype, used as a control, were from R&D Systems (Abingdon, UK). The rabbit polyclonal antibody against myeloperoxidase (MPO) was from DAKO (Carpinteria, CA). The Ready-SET-GO! Mouse IL-17 ELISA kit and the Mouse IL-23 ELISA kit were from eBioscience (San Diego, CA). The HMGB1 ELISA kit II was from Sino-Test Corporation (Tokyo, Japan). Other chemicals were of analytical or the highest grade commercially available.

![FIG. 1. The metabolic pathways of CBZ.](https://academic.oup.com/toxsci/article-abstract/130/1/4/1666677)
CBZ and OXC administration. Male BALB/cCrSlc mice (8–10 weeks old, 22–27 g) were obtained from SLC Japan (Hamamatsu, Japan). Mice were housed in a controlled environment (temperature 23 ± 1°C, humidity 50 ± 10%, and 12-h light/12-h dark cycle) in the institution’s animal facility with ad libitum access to food and water. Animals were acclimatized before use in the experiments. Mice were orally administered CBZ (in corn oil) at a dose of 400 mg/kg for 4 days and 400 to 800 mg/kg on the 5th day. As a control, mice were administered OXC at a dose of 400 mg/kg for 4 days and 800 mg/kg on the 5th day. This dosing regimen is termed “method A” in the following studies. At 1.5, 3, 6, 12, 24, 48, and 72 h after the last administration, the blood and the liver were collected. During a repeated administration study, body weight was recorded. In the single administration study, mice were administered 400 mg/kg or 800 mg/kg CBZ and were sacrificed at 3 h (for assay of the plasma concentration of CBZ and its metabolites) or 24 h (for measurement of the ALT level) after administration. A portion of each excised liver was fixed in 10% formalin neutral buffer solution and used for immunohistochemical staining. The degree of liver injury was assessed by hematoxylin and eosin (H&E) staining, and ALT and AST levels were measured by a DRI-CHEM (Fujifilm). Animal maintenance and treatment were conducted in accordance with the National Institutes of Health Guide for Animal Welfare of Japan, and the protocols were approved by the Institutional Animal Care and Use Committee of Kanazawa University, Japan.

Treatment with Cyp3a inhibitors. One hour before the last CBZ administration, ketoconazole (KTZ; 50 mg/kg in corn oil) or treloandemycin (TAO; 100 mg/kg in corn oil) was injected ip into the mice. The doses of the inhibitors have been used in previous studies (Jin et al., 2009; Pellinen et al., 1994). Blood samples were collected 3 h (for assay of the plasma concentration of CBZ and its metabolites) or 24 h (for measurement of the ALT and AST levels) after the last CBZ administration.

Administration of a TLR4 antagonist and an anti-mouse RAGE antibody. Mice were iv treated with eritoran, a TLR4 antagonist, (50 µg/mouse, dissolved in 0.5 ml sterile saline) or ip treated with an anti-mouse RAGE antibody (100 µg anti-mouse RAGE antibody in 0.5 ml sterile PBS) 6 h after the last CBZ administration using previously described methods (Chavakis et al., 2003; Savov et al., 2005).

Administration of an anti-mouse IL-17 antibody. According to our previous report (Kobayashi et al., 2009), in the neutralization study, mice were ip treated with anti-mouse IL-17 antibody (100 µg anti-mouse IL-17 antibody in 0.5 ml sterile PBS) 6 h after the last CBZ administration. As a control, rat IgG2a was administered (100 µg rat IgG2a in 0.5 ml sterile PBS).

Quantitation of hepatic MPO-positive cells. The infiltration of mononuclear cells was assessed by immunostaining for MPO. A rabbit polyclonal antibody against MPO was used for immunohistochemical staining of the liver as previously described (Kumada et al., 2004). Five visual fields of 400x magnification (0.1 mm² each) were randomly selected from each MPO-immunostained specimen, and a picture was taken with a digital camera (D-33E, OLYMPUS, Tokyo). The average number of MPO-positive mononuclear cells from five randomly selected visual fields was compared among the specimens.

Treatment with PGE1. Nine hours after the last CBZ administration, mice were ip treated with PGE1, (50 µg/mouse, dissolved in 0.5 ml sterile saline). As a control, the vehicle was administered.

Statistical analysis. The data are shown as the means ± SEM. Statistical analyses between multiple groups were performed using one-way ANOVA with Dunnet’s post hoc test for significance between individual groups. Comparisons between two groups were carried out using two-tailed Student’s t-tests. A value of p < 0.05 was considered statistically significant.

RESULTS

Evaluation of CBZ-Induced Liver Injury in BALB/c Mice

CBZ is known to cause hepatotoxicity with inflammation in only a small number of patients. To provide an experimental platform for mechanistic studies of hepatotoxicity, we sought to develop an animal model of CBZ-induced liver injury in mice. Male BALB/c mice were orally administered CBZ at a dose of 400 mg/kg for 5 days. The plasma ALT and AST levels were unaffected by CBZ administration (Fig. 2A). Next, mice were orally administered CBZ at a dose of 800 mg/kg for 5 days, resulting in severe hepatotoxicity in various mice and death in others, presumably due to pharmacological adverse effects during the repeated administration (data not shown).

We then successfully established a dosing regimen for hepatic injury without fatality, named “method A,” consisting of oral administrations at a dose of 400 mg/kg for 4 days and 800 mg/kg on the 5th day. Mice were administered CBZ by method A, and the plasma ALT and AST levels were significantly increased 24 and 48 h after the last CBZ administration (Fig. 2A). These effects were observed in approximately 75% of the mice. In contrast, 25% of the mice showed no hepatotoxicity, and thus, the SEM values were large. The plasma ALT levels were higher than the AST levels, suggesting that tissue damage induced by CBZ administration might occur predominantly in liver. OXC, which was used as negative control, did not induce hepatotoxicity in the same dosing conditions. During the repeated administration study, the body weight of the mice did not change (data not shown).

We altered the dose of CBZ on the 5th day to 400, 600, or 800 mg/kg. No change in the plasma ALT level was observed in mice administered 400 or 600 mg/kg, whereas the dose of 800 mg/kg induced severe liver injury (Fig. 2B). In the single administration study, the plasma ALT level did not change after any of the experimental doses (Fig. 2C).

In the histological evaluation study, focal necrosis and loss of hepatocytes around the central vein were observed 24 h after the last CBZ administration with method A (Fig. 2D). No histopathological differences were observed among no treatment (NT), the lower dose of CBZ-administered (400 mg/kg for 5 days) and OXC-administered mice (Fig. 2D).

Analysis of the Plasma Concentration of CBZ and Its Metabolites

Changes in the plasma concentration of CBZ and its metabolites, CBZ-10,11-epoxide, trans-10,11-dioH CBZ, 2-OH CBZ, and 3-OH CBZ, were measured in mice with CBZ-induced liver injury. The maximum plasma concentrations of CBZ, CBZ-10,11-epoxide, and trans-10,11-dioH CBZ were observed 1.5 h after the last CBZ administration. In contrast, the time of the highest concentration of 3-OH CBZ was 3 h postadministration (Fig. 3A). The plasma concentration of 2-OH CBZ was too low to detect (data not shown). After the peak times, the plasma concentrations of CBZ and the three metabolites decreased dependently of time (Fig. 3A).

Effects of Cyp3a Inhibitors on CBZ-Induced Liver Injury

CBZ induces many drug metabolism enzymes including CYP3A and CYP2B in the liver (Oscarson et al., 2006).
FIG. 2. Time- and dose-dependent changes in plasma ALT and AST levels in CBZ-induced liver injury. Male BALB/c mice were orally administered CBZ by method A (400 mg/kg for 4 days and 800 mg/kg on the 5th day) or 400 mg/kg for 5 days. As the control, mice were administered OXC by method A. At 1.5, 3, 6, 12, 24, 48, and 72 h after the last administration, the blood was collected for assessment of the plasma ALT and AST levels (A). In a dose-dependent study, mice were administered CBZ at a dose of 400 mg/kg for 4 days and 400–800 mg/kg on the 5th day, and blood was collected for assessment of the plasma ALT level 24 h after the last administration (B). In a single administration study, mice were administered CBZ at a dose of 400 mg/kg or 800 mg/kg, and blood was collected 24 h after administration (C). Liver tissue sections were stained with H&E (D). The data are shown as the means ± SEM of the results of the method A group from eight mice and other groups from five mice. Differences compared with the control (0 h) mice were considered significant at *p < 0.05 and **p < 0.01.
FIG. 3. Changes in the plasma concentration of CBZ and its metabolites and plasma ALT and AST levels in CBZ-induced liver injury. Mice were orally administered CBZ by method A. At 1.5, 3, 6, 12, and 24 h after the last CBZ administration, the blood was collected for assessment of CBZ and its metabolites in plasma (A). The data are shown as the means ± SEM of the results from five mice. The plasma ALT and AST levels and the concentration of CBZ and its metabolites were measured after the administration of Cyp inhibitors in mice with CBZ-induced liver injury (B and C). One hour before the last CBZ administration, KTZ (50 mg/kg in corn oil) or TAO (100 mg/kg in corn oil) was ip administered. Blood samples were collected 3 h (for assay of the plasma concentration of CBZ and its metabolites) or 24 h (for measurement of the ALT and AST levels) after the last CBZ administration. The data are shown as the means ± SEM of the results from five mice. Differences compared with the CBZ-alone-administered mice were considered significant at *p < 0.05 and **p < 0.01.
CYP3A metabolizes CBZ into CBZ-10,11-epoxide, 3-OH CBZ, and reactive metabolites in vitro (Lu and Uetrecht, 2008; Pirmohamed et al., 1992b), leading to the hypothesis that CYP3A may be involved in CBZ-induced toxicity. To investigate whether Cyp3a is involved in CBZ-induced liver injury in vivo, mice were treated with the Cyp3a inhibitors KTZ or TAO. Surprisingly, the plasma ALT levels significantly increased after TAO treatment and exhibited an increasing trend after KTZ treatment in CBZ-administered mice. The plasma AST levels also exhibited an increasing trend after KTZ or TAO treatment in CBZ-administered mice (Fig. 3B). Single administration of KTZ (50 mg/kg, ip) or TAO (100 mg/kg, ip) caused no increase in the plasma ALT level 1 h (24.2 ± 6.3 U/l or 27.3 ± 3.7 U/l, respectively) and 25 h (19.8 ± 6.6 U/l or 19.3 ± 5.4 U/l, respectively) after the administration, suggesting KTZ or TAO did not induce hepatotoxicity in the present dosing condition.

The plasma concentrations of CBZ, CBZ-10,11-epoxide, and 3-OH CBZ 3 h after the coadministration of CBZ and KTZ or TAO are shown in Figure 3C. Mice that were administered a single dose of CBZ (800 mg/kg), which caused no hepatotoxicity (Fig. 2C), were used as negative controls. KTZ or TAO treatment significantly increased the plasma concentration of CBZ and significantly decreased the concentration of CBZ-10,11-epoxide, whereas the plasma concentrations of both compounds were significantly increased in mice administered a single dose of CBZ. KTZ exhibited an increasing trend and TAO significantly increased 3-OH CBZ, which exhibited a decreasing trend in mice administered a single dose of CBZ. These data suggest that Cyp3a might be involved in detoxification in CBZ-induced liver injury.

To confirm the Cyp induction by CBZ or OXC with the administration by method A, we measured Cyp3a activity in the microsomes of mice using the metabolism of midazolam as an indicator. The Cyp3a activities were significantly higher in both CBZ- and OXC-administered mice compared with NT mice (Supplementary fig. 1). Because Cyp3a activity was significantly increased in OXC-administered mice, mice were coadministered OXC and TAO, but plasma ALT or AST level was not changed (data not shown).

Changes in GSH Levels and the GSH/Glutathione Disulfide Ratio in the Liver

To investigate whether GSH is involved in detoxification in vivo in mice, time-dependent changes in GSH and glutathione disulfide (GSSG) levels in the liver were measured. The GSH level was the lowest 1.5 h after the last CBZ administration and was significantly decreased at 1.5, 3, 6, 12, and 24 h compared with mice at 0 h (time for the final administration of CBZ). GSSG level exhibited a similar profile (Fig. 4A).

We measured the GSH/GSSG ratio, a biomarker of oxidative stress (Fig. 4A). The ratio of GSH/GSSG was significantly

**FIG. 4.** Time-dependent changes in hepatic GSH, GSSG, and oxidative stress marker levels. Mice were orally administered CBZ by method A. At 1.5, 3, 6, 12, and 24 h after the last CBZ administration, the livers and the plasma were collected for assessment of the hepatic GSH and GSSG levels and GSH/GSSG ratio (A) and the content of plasma protein carbonyls (B). The data are shown as the means ± SEM of the results from five mice. Differences compared with the 0h mice were considered significant at *p < 0.05 and **p < 0.01.
decreased 1.5, 3, and 6 h after the last CBZ administration. In addition, the level of protein carbonyls, which is also a marker of oxidative stress, was significantly increased 1.5 h after the last CBZ administration (Fig. 4B). These results suggested that GSH played a protective role, and oxidative stress is involved in an early stage of CBZ-induced liver injury.

The Expressions of Danger Signals and Their Receptors

It has been reported that ROS elevated the expression levels of danger signals, such as S100A8, S100A9, and HMGB1 (Yao and Brownlee, 2010). To investigate whether danger signals are involved in the onset of inflammation, time-dependent changes in the mRNA expression levels of S100A8, S100A9, HMGB1, TLR2, TLR4, TLR9, and RAGE were measured (Fig. 5A). The mRNA expression levels of S100A8 and S100A9 were time-dependently increased and significantly increased 24 h after the last CBZ administration. The expression of TLR4 was significantly increased at 6 h, and the expression level of RAGE was significantly increased at 12 h postadministration; however, the expression levels of HMGB1, TLR2, and TLR9 were not altered. In OXC-administered mice, the mRNA expression levels of danger signal–related genes were not changed compared with the NT control. It is known that HMGB1 is actively secreted from activated immune cells and is also passively released from necrotic cells (Wang et al., 2004). Thus, the release of HMGB1 is not correlated with the increased expression of hepatic HMGB1 mRNA. The plasma concentration of HMGB1 protein was measured using ELISA, and it was significantly increased at 24 h (Fig. 5B).

To investigate whether TLR4 and RAGE signaling were involved in CBZ-induced liver injury, eritoran, a TLR4 antagonist, or a monoclonal anti-RAGE antibody was administered to the mice. Eritoran or anti-RAGE antibody treatment significantly suppressed the plasma ALT and AST levels (Fig. 5C). These results suggested that TLR4 and RAGE activation might be involved in CBZ-induced liver injury.

The Involvement of Cytokines and Chemokines

To investigate the involvement of inflammatory factors in CBZ-induced liver injury, time-dependent changes in the mRNA expression levels of transcriptional factors, cytokines, and chemokines were measured (Fig. 6A). The expression level of ROR-γt was significantly increased 12 h after the last CBZ administration compared with that of NT mice. T-bet was significantly decreased, and GATA-3 was not altered. The expression levels of IL-6, IL-23 p19, FasL, and macrophage inflammatory protein-2 (MIP-2) were significantly increased compared with those of NT mice, whereas IL-12 p35 and IFN-γ were significantly decreased (Fig. 6A). The expression level of IL-17 mRNA was too low to detect (data not shown). In OXC-administered mice, the mRNA expression levels of cytokines and chemokines were not changed compared with the levels in the NT controls.

The plasma concentrations of IL-17 and IL-23 protein measured by ELISA were significantly increased 24 h after the last CBZ administration (Fig. 6B). The plasma concentration of IFN-γ protein could not be detected at any of the tested time points (data not shown).

To investigate whether IL-17 was involved in CBZ-induced liver injury, we conducted a neutralization study. A monoclonal anti-mouse IL-17 antibody was ip administered 9 h after the last CBZ administration, resulting in significantly reduced plasma ALT and AST levels 24 h after the last CBZ administration (Fig. 6C). These effects were not observed after the administration of an IgG control antibody. In the histopathological evaluation study, anti-mouse IL-17 antibody treatment significantly decreased the number of MPO-positive cells (Fig. 6D). Representative photographs of anti-MPO staining are shown in Supplementary figure 2.

Effects of PGE1 Treatment

PGEs are known to protect against drug-induced and immune-mediated liver injury by downregulating the production of inflammatory cytokines. PGE, inhibited the function of neutrophils (Talpain et al., 1995) and the production of IL-17 (Kobayashi et al., 2009). It was reported that IL-6 and IL-23 induced IL-17 production (Langrish et al., 2005). On the basis of these studies, we hypothesized that PGE, might decrease the production of IL-6 and IL-23 in the present study. PGE, conjugated by α-cyclodextrin was ip injected into mice 9 h after the last CBZ administration according to the method reported previously (Kobayashi et al., 2009). The plasma ALT and AST levels were significantly decreased by PGE treatment in CBZ-administered mice compared with CBZ-alone-administered mice (Fig. 7A). PGE, administration significantly suppressed the hepatic mRNA expression of IL-6, IL-23p19, and MIP-2 (Fig. 7B). The plasma concentrations of IL-17 and IL-23 proteins were also decreased (Fig. 7C).

DISCUSSION

Advances in the understanding of the mechanisms of drug-induced liver injury have been hampered by the lack of proper animal models. Mouse models of APAP-induced liver injury are the widespread model, but this model alone cannot encompass the entire spectrum of the clinical and mechanistic features of drug-induced liver injury in patients. In the present study, we developed a mouse model of CBZ-induced liver injury. This model provided novel mechanistic information, including aspects of drug metabolism and inflammation in the pathogenesis of CBZ-induced liver injury.

Subacute toxicity study demonstrated that no evidence of hepatotoxicity was observed after repeated administrations of CBZ (200 mg/kg, orally) once daily for 24 weeks to mice (Novartis Pharma Co., 2011). After investigating many different dosing conditions, we found that CBZ at a dose of 400 mg/kg for 4 days and 800 mg/kg on the 5th day (method A) induced prominent hepatotoxicity, but 400 mg/kg for 5 days did not (Fig. 2A). The maximum plasma concentration of CBZ...
FIG. 5. Time-dependent changes in the mRNA expression levels of danger signal–related genes in the liver of CBZ-administered mice (A) and the plasma HMGB1 protein levels in CBZ-administered mice (B). Mice were orally administered CBZ by method A, and the livers and plasma were collected 0, 6, 12, and 24 h after the last CBZ administration for assessment of the expression levels of the hepatic mRNA or the plasma protein levels. As the control, mice were orally administered OXC by the same method A, and the livers and plasma were collected 24 h after the last OXC administration. The expression of hepatic mRNA was normalized to that of β-actin. The effects of the administration of eritoran, a TLR4 antagonist, or anti-RAGE-monoclonal antibody on CBZ-induced liver injury (C). Mice were iv administered eritoran (50 µg/mouse in 0.1 ml sterile saline) or ip anti-mouse RAGE antibody (100 µg anti-RAGE antibody in 0.5 ml sterile PBS) 6 h after the last CBZ administration. Blood samples were collected 24 h after the last CBZ administration. The data are shown as the means ± SEM of the results of the time-dependent study from five mice, the method A group from eight mice, and the other groups from five mice. Differences compared with the 0 h mice were considered significant at *p < 0.05 and **p < 0.01, and differences compared with the CBZ-alone-administered mice were considered significant at †p < 0.05.
FIG. 6. Time-dependent changes in the hepatic mRNA expression levels and plasma protein levels of proinflammatory cytokines and chemokines in CBZ-induced liver injury (A and B). Mice were orally administered CBZ by method A, and the livers and plasma were collected 0, 6, 12, and 24 h after the last CBZ administration for assessment of the expression levels of the hepatic mRNA by real-time RT-PCR or the plasma protein levels by ELISA. Mice were orally administered OXC by the same method A, and the livers and plasma were collected 24 h after the last administration. The expression of hepatic mRNA was normalized to that of β-actin. The data are shown as the means ± SEM of the results from five mice. Differences compared with the NT mice were considered significant at *p < 0.05, **p < 0.01, and ***p < 0.001. The neutralization study of IL-17 in CBZ-induced liver injury (C). Mice were ip administered an anti-mouse IL-17 antibody (100 µg anti-mouse IL-17 antibody in 0.5 ml sterile PBS) 6 h after the last CBZ administration. As a control, rat IgG2a was administered (100 µg rat IgG2a in 0.5 ml sterile PBS). The number of MPO-positive cells in CBZ-alone-administered mice or CBZ and anti-IL-17-administered mice (D). The infiltration of mononuclear cells was assessed by immunostaining for MPO. The data are shown as the means ± SEM of the results of the method A group from eight mice and the other group from six mice. Differences compared with the CBZ- and IgG-administered mice were considered significant at †p < 0.05 and ††p < 0.01.
1.5 h after the last administration (43.8 ± 20.7 μM) was nearly equal to the steady-state human plasma concentration of CBZ (Eichelbaum et al., 1975). In clinical therapeutics, the dosage of CBZ is gradually increased for the maintenance of the therapeutic plasma concentration because CBZ is a potent inducer of microsomal drug metabolism (Oscarson et al., 2006). In fact, CBZ induced the Cyp3a enzyme activity in our novel mouse model (Supplementary fig. 1). Based on our findings, it was suggested that the escalation of the dosage for the maintenance of the therapeutic plasma concentration caused the risk for CBZ-induced liver injury. In addition, the single administration of CBZ at a dose of 800 mg/kg did not induce hepatotoxicity (Fig. 2C), suggesting that repeated administration is necessary to cause CBZ-induced liver injury. Due to the presence of a specific autoantibody directed against a human liver microsomal protein in a patient who had severe hepatotoxicity with CBZ (Pirmohamed et al., 1992a), the induction of drug metabolism enzymes and/or the generation of antibodies directed against CBZ-protein conjugates during repeated administration may be involved in CBZ-induced liver injury. The dosing method of CBZ in the present study might be useful for the development of animal models for other drug-induced liver injury.

In the histopathological study, remarkable hepatic necrosis and loss of hepatocytes, especially around the central vein, were observed in the mice administered CBZ by method A, and these effects were similar to APAP-induced liver injury (Antoine et al., 2009). Because Cyps are mainly expressed around the central vein in the liver, this observation suggested that Cyps may be involved in CBZ-induced liver injury. CBZ-associated severe hepatotoxicity takes the following two forms: (1) a...
hypersensitive reaction in the form of granulomatous hepatitis that present with fever and abnormal liver functions and (2) an acute hepatitis and hepatocellular necrosis with inflammation (Björnsson, 2008; Björnsson and Olsson, 2005). The mouse model in the present study may fit the latter form.

Changes in the plasma concentration of 3-OH CBZ suggested a certain role for the metabolite in CBZ-induced liver injury in the present study. Additionally, 2-OH CBZ, which is also a potential reactive metabolite, was not detected in plasma, which is coincident with a report that 2-OH CBZ is generated to a much lesser extent than 3-OH CBZ in vitro (Pearce et al., 2002). Based on these results, 2-OH CBZ may not likely to be involved in CBZ-induced liver injury in vivo. It has been reported that 3-OH CBZ, a reactive metabolite produced by a variety of CYPs, induced ROS causing mitochondrial dysfunctions (Pearce et al., 2008), which causes the suppression of GSH levels and the alteration of oxidative stress markers. CYP3A4 and CYP2B6 are largely responsible for the formation of 3-OH CBZ in humans (Pearce et al., 2002), and these enzymes are induced by CBZ treatment. Therefore, repeated administration of a high dose of CBZ may cause the elevation of 3-OH CBZ. Because there is no direct evidence that 3-OH CBZ is a reactive metabolite or is related to the production of reactive metabolite(s), further studies are needed to examine these possibilities. In addition, 3-OH CBZ cannot be used directly for accessing hepatotoxicity due to different in vivo distribution and pharmacokinetics compared with CBZ. Treatment with Cyp3a inhibitors exacerbated the hepatic injury caused by CBZ (Fig. 3B), suggesting that the main function of Cyp3a is detoxification in CBZ-induced liver injury. Contrary to this result, Pirmohamed et al. (1992b) demonstrated that KTZ reduced the cytotoxicity of bioactivated CBZ by liver microsomes from phenobarbital-administered mice. The difference in the results between in vitro and in vivo systems is not unusual and should be carefully evaluated, especially in cases involving enzyme induction.

It has recently been reported that ROS induced the expression of the ligands of TLR4 and RAGE (Yao and Brownlee, 2010), and inflammation in the liver through the activation of TLR4 or RAGE is involved in APAP-induced liver injury (Antoine et al., 2009). On the basis of these experimental results, we demonstrated that the activation of TLR4 and RAGE by ROS is the essential factor in relation to the drug metabolism and inflammation in CBZ-induced liver injury. Mu et al. (2011) demonstrated that the activation of TLR4 prompted the generation of Th17-associated cytokines. Therefore, the activation of TLR4 and RAGE might induce the generation of Th17-associated cytokines, resulting in inflammation in the liver in the present study.

IL-17 induced by IL-6 and IL-23 stimulates the production of CXC-chemokines (such as MIP-2 and keratinocyte-derived chemokine) and activates neutrophils (Langrish et al., 2005; Steinman, 2007). IL-17 was involved in the pathogenesis of various autoimmune diseases and immune-mediated hepatotoxicity in mice (Kobayashi et al., 2009, 2010). These lines of evidence prompted us to confirm the involvement of IL-17 in CBZ-induced liver injury. The neutralization of IL-17 reduced the plasma ALT and AST levels and MPO-positive cells in the liver (Figs. 6C and D), which suggested that infiltration of neutrophils into the liver via IL-17 was involved in CBZ-induced liver injury.

FIG. 8. A proposed mechanism of CBZ-induced liver injury. CBZ is metabolized in hepatocytes by Cyp3a, the produced reactive metabolite(s) induce ROS production in macrophages, and then danger signals released from macrophage activate TLR4 and RAGE. The activated TLR4 and RAGE lead to the secretion of proinflammatory cytokines and chemokines, which result in inflammation in the liver. The necrotic hepatocytes secrete the ligands of TLR4 and RAGE, which induce further inflammation in the liver.
PGE, treatment 9 h after the last CBZ administration ameliorated CBZ-induced liver injury (Fig. 7A), and PGE, suppressed the production of IL-6 and IL-23, resulting in the decrease of plasma IL-17 concentration (Figs. 7B and C). PGE, inhibited superoxide production by neutrophils in vitro (Talpain et al., 1995) and had a protective effect against halothane-induced liver injury by the neutralization of IL-17 in mice (Kobayashi et al., 2009). Additionally, PGE had protective effects on livers suffering from ischemia/reperfusion injury and decreased the plasma IL-6 levels in humans (Sugawara et al., 1998). PGE, could be used for pharmacotherapy of CBZ-induced liver injury in clinical practice.

CBZ causes not only hepatotoxicity but also cutaneous drug reactions, including maculopapular eruption, hypersensitivity syndrome, Stevens-Johnson syndrome, and toxic epidermal necrolysis. It is well known that human leukocyte antigen alleles are strongly associated with CBZ-induced cutaneous adverse drug reactions (Hung et al., 2006). In the present study, cutaneous drug reactions were not observed in the mouse model of CBZ-induced liver injury. Thus, the development of an experimental animal model of CBZ-induced cutaneous adverse drug reactions is still needed.

In conclusion, we developed a mouse model of CBZ-induced liver injury. Based on the results of the present study, the proposed mechanisms are summarized in Figure 8. Information resulting from our novel mouse model could eventually be applied to preclinical drug development.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING


REFERENCES


P450 responsible for the formation of 2- and 3-hydroxylated metabolites. 
*Drug Metab. Dispos.* **30**, 1170–1179.


