INTRODUCTION

Idiosyncratic drug toxicity (IDT) is one of the serious concerns of pharmaceutical companies. Even though the incidence is as low as 1/1,000 to 1/100,000, IDT is known to often cause serious disorders such as severe hepatotoxicity, agranulocytosis, neutropenia, Stevens-Johnson syndrome, or other illnesses. IDT is unpredictable, bears no obvious relationship to its dose, demonstrates inconsistent temporal patterns with regard to drug exposure, and is typically unrelated to the intended pharmacological effect (Park et al., 2000; Roth et al., 2003; Waring and Anderson, 2005). In addition, IDT is often found in the final phase of a clinical study or after marketing since IDT cannot be evaluated in non-clinical safety studies using healthy animals (Kaplowitz, 2005; Baillie, 2006). If this occurs, the relevant pharmaceutical company has to shoulder a great burden and consequently might be forced to discontinue the development of a drug or to withdraw it from the market. Therefore, it is an urgent task for pharmaceutical companies to establish the approach and an assessment method to detect IDT in the earliest stages of drug development.

The mechanisms of IDT are not fully understood despite the large number of drugs associated with it. Several conventional hypotheses have been proposed regarding the mechanisms of IDT onset, suggesting that a metabolic activation of drugs to reactive metabolites and a specific immune response to their covalent binding to tissues are involved (Walgren et al., 2005; Uetrecht, 2008;...
Pirmohamed et al. (2002). However, no experimental evidence supporting these hypotheses has been obtained as yet due to lack of appropriate animal models.

Studies to elucidate the mechanisms of IDT onset are under way especially regarding the usefulness of various animal models including models of immune function modified by concomitant use of lipopolysaccharide (LPS). LPS is a toxic component of the cell wall of Gram-negative bacteria and is widely present in the digestive tract of humans and animals. In experimental animals administered LPS, this activates the immune system and inflammatory cells leading to amplification of the inflammatory response by releasing various cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6 (Beutler and Rietschel, 2003; Tanaka et al., 2010; Mohamadin et al., 2011). As to models using LPS, it has been reported that co-treatment with a compound known to induce IDT and a low dose of LPS caused a disorder similar to IDT in rats (Buchweitz et al., 2002; Waring et al., 2006; Luyendyk et al., 2006; Deng et al., 2006; Zou et al., 2010). For example, co-administration of non-hepatoxic doses of LPS and the antipsychotic drug chlorpromazine to rats results in liver damage that resembles chlorpromazine-induced IDT (Buchweitz et al., 2002). Trovafloxacin, a fluoroquinolone antibiotic known to induce IDT, also interacts with LPS, resulting in rat hepatotoxicity that is qualitatively different at the histologic level from that occurring in rats treated with high doses of LPS alone. In contrast, levofloxacin, another fluoroquinolone antibiotic without IDT, does not result in rat hepatotoxicity (Waring et al., 2006). Ranitidine, the histamine 2 (H2)-receptor antagonist, is also known to induce idiosyncratic hepatotoxicity and induces liver injury in the LPS model even though ranitidine itself is not hepatotoxic in rats. In addition, famotidine, another H2-receptor antagonist without IDT, does not result in liver injury in the LPS model (Luyendyk et al., 2006). These reports indicate that liver injury induced in the LPS model does not result from in general additive or synergistic effects between two compounds. Deng et al. (2006) reported that the co-administration of LPS and diclofenac (DCF) to rats induced liver injury via activation of the immune system and therefore the LPS model might be useful to predict IDT.

DCF is extensively used all over the world as a non-steroidal anti-inflammatory drug, but it is well known to induce IDT (Boelsterli, 2003; Walker, 1997). Although the apparent incidence of severe DCF-induced hepatic adverse reactions is quite low (one to two cases per million prescriptions or six to 18 cases/100,000 persons per year), the large number of patients taking DCF makes the absolute number of cases impressive (Walker, 1997). In addition, severe injury leading to liver transplantation occurred in a large proportion of the reported cases of DCF-induced hepatotoxicity (Lewis, 2003).

It has been postulated lately that inflammatory stress induced by exogenous or endogenous inflammmagens is a susceptibility factor involved in the mechanism of IDT onset (Deng et al., 2009; Shaw et al., 2010). In the case of DCF, many patients have inflammatory diseases including rheumatoid arthritis and osteoarthritis, etc (Banks et al., 1995). In fact, there is a report that osteoarthritis increased the risk of DCF-induced idiosyncratic hepatotoxicity (Banks et al., 1995). It is possible that an LPS model may reflect the clinical environment of patients as LPS induces an inflammatory response.

On the other hand, bioactivation of drugs to reactive metabolites and their covalent binding to cellular macromolecules are believed to be involved in clinical adverse events, including IDT. It is known that several reactive metabolites are generated during DCF metabolism, suggesting their involvement in IDT. Both the 4′- and 5-hydroxy metabolites of DCF (4′-hydroxydiclofenac (4′-OH-DCF) and 5-hydroxydiclofenac (5-OH-DCF)) have the potential to be further oxidized to a p-benzoquinone imine. Quinone imines are well-known reactive metabolites that undergo covalent binding to non-protein or protein sulfhydryl groups due to their thiol reactivity (Boelsterli, 2003). In addition, arene-oxide is reportedly the primary intermediate of 4′-OH-DCF and 5-OH-DCF and may induce irreversible inactivation through its covalent binding to a xenobiotic-metabolizing enzyme cytochrome P450 (CYP) in the liver (Masubuchi et al., 2001, 2002). Acyl glucuronides of DCF are also potentially protein-reactive intermediates per se because of their rather labile ester bond (Grillo et al., 2003).

Based on the hypotheses and evidence of liver injury in rats co-treated with LPS and a compound known to induce IDT, we postulated that an LPS treatment not only activates the immune system but also may affect covalent binding of reactive drug metabolites to cellular macromolecules. In the present study, the effects of LPS on reactive drug metabolites were investigated in rats by use of a model compound DCF that is well known to produce several reactive metabolites and to induce IDT.

**MATERIALS AND METHODS**

**Reagents**

LPS, DCF and 4′-OH-DCF were purchased from Sigma Chemical Co. (St. Louis, MO, USA). LPS derived from Escherichia coli 055:B5 (lot number 127K4048).
with not less than 500,000 EU (endotoxin units)/mg was used in these studies. 5-OH-DCF was purchased from Toronto Research Chemicals Inc. (Ontario, Canada). All other reagents and solvents were of the highest grade commercially available.

Animals and treatments

Male Sprague-Dawley (Crl:CD(SD)] rats (310 to 390 g, 10 weeks of age) raised under specific-pathogen-free conditions, were obtained from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). They were housed individually in stainless steel breeding cages in a barrier-system room controlled for temperature (21-25°C), humidity (40-70%), and lighting (a 12-hr light/dark cycle). They were allowed access to a commercial diet (CE-2; CREA Japan, Inc., Tokyo) and water ad libitum. After one week of quarantine, the animals were used in this study.

Animal experiments were performed according to experimental design of Deng et al. (2006) with slight modifications. The animals were intravenously administered LPS (1 or 10 μg/2 ml saline/kg) and 2 hr later they were intraperitoneally administered diclofenac (20 mg/4 ml saline/kg). The control animals received saline alone in the same fashion.

In experiment (Exp.) 1, rats (n = 4-7/group) were treated with saline (i.v.)/saline (i.p.), saline (i.v.)/DCF, LPS (1 μg/kg)/saline (i.p.), LPS (10 μg/kg)/saline (i.p.), LPS (1 μg/kg)/DCF or LPS (10 μg/kg)/DCF and euthanized by exsanguination 24 hr after the administration of DCF or saline (i.p.); blood was collected from the abdominal aorta in the presence of heparin sodium as an anticoagulant, and the liver was removed. The plasma samples separated from the collected blood were used for analysis of ALT and AST activities and the isolated livers were used for histopathological assessment and preparation of microsomes used for the in vitro assays of total P450 content, testosterone hydroxylation activity, DCF hydroxylation activity and dansyl glutathione (dGSH) trapping assay. In Exp. 2, rats (n = 5/group) were treated with saline (i.v.)/DCF or LPS (10 μg/kg)/DCF and blood samples of approximately 0.2 ml for toxicokinetic analyses were collected from the tail vein, without anesthesia, using heparin sodium as the anticoagulant, at 0.5, 1, 2, 4, 6, 20 and 24 hr after the administration of DCF. In Exp. 3, rats (n = 4/group) were treated with saline (i.v.)/DCF, LPS (1 μg/kg)/DCF or LPS (10 μg/kg)/DCF and euthanized by exsanguination 24 hr after the administration of DCF and the liver was collected to evaluate the extent of covalent binding of DCF reactive metabolites to hepatic tissues. In Exp. 4, rats (n = 4-6/group) were treated with saline (i.v.)/saline (i.p.), LPS (10 μg/kg)/saline (i.p.), saline (i.v.)/DCF or LPS (10 μg/kg)/DCF and euthanized by exsanguination 24 hr after the administration of DCF or saline (i.p.) and the liver was collected to evaluate the hepatic GSH level.

In our preliminary studies, doses up to 40 mg/kg (i.p.) of DCF alone or 10 μg/kg (i.v.) of LPS alone did not cause elevation in the plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. Furthermore, a high-dose treatment with DCF alone at 100 mg/kg (i.p.) induced severe gastrointestinal toxicity but not liver injury (data not shown). The DCF-induced gastrointestinal toxicity was not observed in any treated groups in this study.

All experiments using laboratory animals were accurately performed in accordance with the animal welfare bylaws of the Safety Research Laboratory, Kissei Pharmaceutical Co., Ltd. The Institutional Animal Care and Use Committee in our institution oversaw animal management during these experiments. Our institution is fully accredited by the Health Science Center for Accreditation of Laboratory Animal Care and Use.

Plasma ALT and AST activities and histopathological assessment in rats treated with LPS/DCF

Plasma ALT and AST activities were determined by the standard methods of the Japan Society of Clinical Chemistry (JSCh) using an automatic analyser 7180 (Hitachi High-Technologies, Tokyo, Japan). The left lateral lobes, the left medial lobes and the right lateral lobes of the liver obtained in Exp. 1 were fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned at 3 μm-thickness, and stained with hematoxylin-eosin for microscopic examination.

In vitro assay using hepatic microsomes prepared from rats treated with LPS

A hepatic microsomal fraction was prepared from the left lateral lobes of the liver isolated from groups treated with saline (i.v.)/saline (i.p.), LPS (1 μg/kg)/saline (i.p.) and LPS (10 μg/kg)/saline (i.p.) in Exp. 1 according to the method of differential centrifugation described by Degawa et al. (1989). The microsomal samples were stored as suspensions in 1.15% potassium chloride solution at -80°C. Microsomal protein concentrations were determined by Lowry’s method (1951).

Total P450 content of hepatic microsomes was quantified from the carbon monoxide difference spectrum of dithionite-reduced proteins between 450 nm and 490 nm, using an extinction coefficient of 91 mM⁻¹ cm⁻³ as described by Omura and Sato (1964).
Testosterone hydroxylation activity in hepatic microsomes was determined by measuring 6β-, 16α- and 16β-hydroxytestosterone resulting from hydroxylation of testosterone according to a published high-performance liquid chromatography (HPLC) method (Imaoka et al., 1989; Kishida et al., 2008) at 1 mM testosterone (testosterone, hydroxytestosterone; Sigma Chemical Co., St. Louis, MO, USA). The 6β-, 16α- and 16β-testosterone hydroxylation activities are known as indicators of the enzyme activity of CYP3A, CYP2C and CYP2B, respectively, which are major CYP subfamilies.

DCF hydroxylation activity in hepatic microsomes was determined by measuring 4'-OH-DCF and 5-OH-DCF according to a published HPLC method (Masubuchi et al., 1989; Kishida et al., 2008) at 1 mM testosterone (testosterone, hydroxytestosterone; Sigma Chemical Co., St. Louis, MO, USA). The 6β-, 16α- and 16β-testosterone hydroxylation activities were known as indicators of the enzyme activity of CYP3A, CYP2C and CYP2B, respectively, which are major CYP subfamilies.

Toxicokinetics of DCF in rats treated with LPS/DCF

Fifty μl of plasma obtained in Exp. 2 was loaded with the internal standard onto a solid-phase extraction column (OASIS® HLB μElution Plate; Waters, Milford, MA, USA), which was preconditioned with methanol followed by water. The plate was washed with water, and the analytes were eluted with 25 μl of acetonitrile/formic acid (95:5, v/v). Toxicokinetic parameters (AUC0-24 hr, T_max, C_max, T1/2) of DCF and its metabolites 4'-OH-DCF and 5-OH-DCF were calculated by Phoenix™ WinNonlin® Ver.6.1 (Pharsight Corporation, Cary, NC, USA). AUC0-24 hr is the area under the plasma concentration-time curve from 0 to 24 hr after dosing, C_max is the maximum plasma concentration, T_max is the time to reach Cmax, and T1/2 is half-life of analyte.

In vivo covalent binding of DCF to hepatic tissues in rats treated with LPS/DCF

Approximately 0.5 g of the left lateral lobes of the liver obtained in Exp. 3 was minced in 0.5 ml of PBS, and extensively washed with 2 ml of methanol/ ether (3:1). Washed pellets obtained after centrifugation at 400 × g for 5 min were submitted to alkaline hydrolysis (1 mol/l KOH at 80°C for 3 hr). After adding 5% trifluoroacetic acid in acetonitrile and tolmetin as the internal standard and centrifuging at 1,500 × g for 5 min, the supernatant was dried at 60°C under a stream of nitrogen gas and the residue was dissolved in a mobile phase. After filtration of the final solution, an aliquot of 10 μl was analyzed. None of the analytes were detected in samples extracted from washed pellets before alkaline hydrolysis.

Bioanalysis of DCF and its metabolites

4'-OH-DCF and 5-OH-DCF

DCF, 4'-OH-DCF and 5-OH-DCF in hepatic tissues or plasma were determined by a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system including the following: a Prominance UFLC (Shimadzu Corporation, Kyoto, Japan) and API3200 MS/MS detector (Applied Biosystems/MDS SCIEX, Ontario, Canada). The separation of analytes was achieved on an L-column2 ODS (3 μm, 2.1 × 100 mm, Chemicals Evaluation and Research Institute, Tokyo) with 10 mM ammonium bicarbonate/acetonitrile (27:8, isocratic mode) at a flow rate of 0.35 ml/min. Monitored pairs of m/z values were 296 > 214 (DCF), 312 > 230 (4'-OH-DCF elution time: 3.1 min), 5-OH-DCF (elution time: 4.6 min) and 258 > 119 (tolmetin, internal standard) in positive ionization mode.
Hepatic GSH level in rats treated with LPS
Approximately 0.1 g of the left lateral lobes of liver obtained in Exp. 4 was homogenized in 5 vol/g (wet weight) of 5% 5-sulfosalicylic acid. Tissue homogenates were centrifuged at 8,000 × g for 10 min at 4°C. The assay for total GSH was made using a total glutathione quantification kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan).

Data analyses
All data are shown as means ± standard deviations. One-way analysis of variance (ANOVA) was used for statistical analysis of the results. P values less than 0.05 were considered significant.

RESULTS
Plasma ALT and AST activities and histopathological assessment in rats treated with LPS/DCF
Neither LPS nor DCF altered plasma ALT and AST activities when given alone at the dose. However, co-treatment with LPS and DCF caused a significant increase in plasma ALT and AST activities when the dose of LPS was increased. Though there were few cases, a high response was observed in plasma ALT and AST activities in the group co-treated with LPS (10 μg/kg, i.v.) and DCF (20 mg/kg, i.p.), which contributed to variation in those values (Fig. 1).

Livers from saline-treated and DCF (20 mg/kg, i.p.)-treated rats had no or minimal histopathological changes (Figs. 2A and 2B). Treatment with LPS (10 μg/kg, i.v.) alone caused slight-to-moderate infiltration of inflammatory cells into Glisson’s sheath that consisted mainly of neutrophils and mononuclear cells (Fig. 2C). Co-treatment with LPS (10 μg/kg, i.v.) and DCF (20 mg/kg, i.p.) caused multifocal midzonal necrosis of hepatocytes in the few cases that had high plasma ALT and AST activities. Although the infiltration of inflammatory cells into Glisson’s sheath related to LPS treatment was also observed, the degree was almost the same as that observed with LPS (10 μg/kg, i.v.) alone (Fig. 2D).

In vitro assay using hepatic microsomes prepared from rats treated with LPS
The effect of LPS on hepatic CYP activity in rats was investigated at a non-hepatotoxic dose of LPS (1 or 10 μg/kg, i.v.). The total P450 content in hepatic microsomes prepared from LPS-treated rats was significantly decreased (LPS 1 μg/kg: 0.66 times, LPS 10 μg/kg: 0.32 times) as compared with the control (Fig. 3A). Furthermore, testosterone hydroxylation activity in the in vitro metabolism study using hepatic microsomes decreased significantly (6β-hydroxylation; LPS 1 μg/kg: 0.47 times, LPS 10 μg/kg: 0.32 times, 16α-hydroxylation; LPS 1 μg/kg: 0.66 times, LPS 10 μg/kg: 0.38 times, 16β-hydroxylation; LPS 1 μg/kg: 0.65 times, LPS 10 μg/kg: 0.37 times).
kg: 0.46 times) (Fig. 3B).

The effect of LPS on 4'-hydroxylation and 5-hydroxylation activities of DCF was investigated using hepatic microsomes prepared from LPS-treated rats. As in the case of total P450 content and testosterone hydroxylation activity, the 4'-hydroxylation activity (LPS 1 μg/kg: 0.53 times, LPS 10 μg/kg: 0.30 times) and 5-hydroxylation activity (LPS 1 μg/kg: 0.48 times, LPS 10 μg/kg: 0.25 times) of DCF demonstrated a significant decrease compared with the control (Fig. 4A). Furthermore, to investigate whether the effect of LPS on DCF hydroxylation in rat hepatic microsomes was the result of direct or indirect reactions, LPS was added at final concentrations of 0.5 to 50 μg/ml to the reaction mixture including the control microsomes prepared from saline-treated rats. As a result, no change was observed in DCF hydroxylation activity after the addition of LPS even at a concentration as high as 50 μg/ml (Fig. 4B), indicating that LPS does not have direct action on the hydroxylation metabolism of DCF and that the mechanism does not involve a metabolic competition between LPS and DCF.

As the next step, to investigate the effect of LPS on the generation of DCF reactive metabolites, dGSH was added as a trap agent of reactive metabolites to a reaction mixture that included hepatic microsomes prepared from LPS-treated rats. Representative chromatograms are shown in Fig. 5A. The generation of dGSH adducts of DCF also demonstrated a marked decrease (LPS 1 μg/kg: 0.18 times, LPS 10 μg/kg: 0.09 times) along with the increase of LPS (Fig. 5B). Other peaks of dGSH adducts on the chromatograms were not evaluated because they were very minor and did not clearly change in relation to LPS treatment.
Toxicokinetics of DCF in rats treated with LPS/DCF

The series of in vitro test results suggested the influence of LPS on the toxicokinetics of DCF. Accordingly, the plasma concentrations of DCF as well as of 4’-OH-DCF and 5-OH-DCF were determined when DCF was administered with or without LPS. Co-treatment caused a delayed elimination of 5-OH-DCF from plasma as compared with DCF alone, whereas the plasma concentration-profiles of DCF and 4’-OH-DCF did not change (Table 1). Plasma concentrations of 5-OH-DCF were higher from 4 hr onward after dosing of DCF after LPS/DCF co-treat-
Fig. 4. Influence of LPS on diclofenac hydroxylation activity in rat hepatic microsomes. (A) Diclofenac hydroxylation activity in hepatic microsomes from saline-treated (control) and lipopolysaccharide (LPS, 1 or 10 μg/kg, i.v.)-treated rats. (B) Diclofenac hydroxylation activity in a reaction mixture including LPS at final concentrations of 0, 0.5, 5 and 50 μg/ml. Data are shown as fold changes to the control and as means ± S.D. (n = 4). Asterisks indicate statistically significant difference (* P < 0.05, ** P < 0.01, vs control).
Effect of LPS on diclofenac reactive metabolites in rats

Fig. 5. Representative chromatograms of diclofenac incubated with rat hepatic microsomes and dansyl glutathione (dGSH) (A), and diclofenac adducts to dGSH in hepatic microsomes from saline-treated (control) and lipopolysaccharide (LPS, 1 or 10 μg/kg, i.v.)-treated rats (B). Data are shown as fold changes to the control and as means ± S.D. (n = 4). Asterisks indicate statistically significant difference (** P < 0.01, vs control).
In vivo covalent binding of DCF to hepatic tissues in rats treated with LPS/DCF

We investigated the effect of LPS on the amount of DCF reactive metabolites adducts in liver isolated at 24 hr after co-treatment with LPS/DCF. Although the adducts under in vivo conditions were assumed to be decreased by LPS treatment based on the in vitro test results (Fig. 5B), no influence of LPS was observed in the amount of DCF adducts (Fig. 7A) and 4’-OH-DCF adducts (Fig. 7B). In fact, 5-OH-DCF adducts demonstrated a significant increase as the LPS dose was increased (LPS 1 μg/kg: 2.2 times, LPS 10 μg/kg: 3.4 times) in comparison with DCF alone (Fig. 7C).

Hepatic GSH level in rats treated with LPS

To interpret the fact that 5-OH-DCF adducts increased as the plasma concentration of DCF increased, we further determined the GSH level in the liver isolated after treatment with LPS and/or DCF. Compared with the vehicle control, the decrease of GSH (0.49 times) reached statistical significance only in the liver of rats co-treated with LPS/DCF (Fig. 8).

DISCUSSION

DCF is well known to induce IDT (Boelsterli, 2003). However, there remains much to be elucidated about the onset mechanism even though various hypotheses have been proposed. One of the reasons is that it is difficult to reproduce the idiosyncratic liver injury induced by DCF in healthy experimental animals even if a high dose of DCF is used. The verification using an appropriate animal model is of great importance. In this study, we demonstrated that in rats co-treatment with LPS and DCF at a non-hepatotoxic dose induced liver injury associated with increased plasma ALT and AST activities. Furthermore, we found an increase of adducts of DCF reactive metabolites via 5-OH-DCF to hepatic tissues, which may play an important role in the onset of IDT.

The hypothesis that IDT arises from a specific immune response to a hapten formed by covalent binding of drugs or their reactive metabolites to hepatic tissues is widely accepted. This hypothesis has not changed since 1987 although several other hypotheses have been added, for example, the danger hypothesis and the pharmaceutical interaction hypothesis (Uetrecht, 2008). In a rat LPS model, liver injury is likely caused by a specific immune-mediated reaction to drug-protein conjugates. This specific immune-mediated reaction could be stimulated by LPS-induced inflammatory responses with amplification of pro-inflammatory cytokines, etc., which are danger signals to produce an immune response to an antigen on the basis of the danger hypothesis (Pirmohamed et al., 2002). In this study, the LPS-induced danger signals might have contributed to the individual variation in responsiveness to LPS/DCF co-treatment. Individual differences in the inflammatory responses to LPS are well known and have been suggested to depend on the variation of Toll-like receptor expression level (Jaekal et al., 2007).

Hepatic CYP enzymes are involved in the generation of DCF reactive metabolites in rats and humans (Masubuchi et al., 2001, 2002). These enzymes oxidize DCF main-
Effect of LPS on diclofenac reactive metabolites in rats

![Fig. 6. Plasma concentration-profiles of 5-hydroxydiclofenac after dosing of diclofenac (DCF, 20 mg/kg, i.p.) with or without pre-treatment with lipopolysaccharide (LPS, 10 μg/kg, i.v.).](image)

Although the time lag up to the onset of CYP down-regulation may have contributed. Moreover, regarding the delayed elimination of 5-OH-DCF from plasma, the decreased clearance by LPS-induced alterations in blood flow (Kim et al., 2009), transporters or phase II enzymes (Yano et al., 2010) may explain the differences observed between the results of in vitro and in vivo experiments. For example, LPS is known to downregulate the multidrug resistance-associated protein 2 (Mrp2) that mediates canalicular secretion of several amphiphilic organic anions including glucuronides and GSH conjugates (Yano et al., 2010). Further investigation is required as to the influence of CYP down-regulation in vivo, but it is important to mention that the results of in vivo studies demonstrated that LPS/DCF co-treatment affected the elimination pathway of DCF via 5-OH-DCF and increased the amount of 5-OH-DCF adducts to hepatic tissues.

GSH is an important cellular component that is crucial for the cellular homeostasis of redox potential and a natural defense against oxidative stress, and also serves as primary intracellular nucleophile. It reacts with many known reactive metabolites, and the resultant GSH conjugation is usually nontoxic and readily excreted. We investigated the effect of LPS/DCF co-treatment on the GSH level in rat hepatic tissues and demonstrated that the hepatic GSH level was decreased by the co-treatment. These findings are supported by recent reports (Mohamadin et al., 2011; Zou et al., 2010) and considered attributable to the oxidative stress generated by co-treatment with LPS. Shen et al. (1999) have reported that covalent binding of reactive metabolites of 5-OH-DCF to human liver microsomes was inhibited by GSH. Based on this finding, the decreased GSH level in hepatic tissues is considered to imply a reduced detoxication reaction of GSH with 5-OH-DCF reactive metabolites, resulting in increased adducts of the reactive metabolites to hepatic tissues. However, further studies are needed to elucidate the reasons why LPS/DCF co-treatment affected the plasma elimination and covalent binding of 5-OH-DCF only, but not 4'-OH-DCF and DCF. One of the possible reasons is that the reactivity of 5-OH-DCF reactive metabolites to GSH may be much higher than that of others; in other words, 5-OH-DCF reactive metabolites may be more sensitive to decreased GSH conditions.

DCF is more cytotoxic to drug-metabolizing cells (rat and human primary cultured hepatocytes) than to non-metabolizing cell lines (HepG2, FaO) and its in vitro cytotoxicity correlates well with an increase in the formation of 5-OH-DCF and the subsequent metabolite N,5-dihydroxydiclofenac that can inhibit ATP synthesis (Bort et al., 1999). In addition, adducts of 5-OH-DCF, but not...
Fig. 7. *In vivo* covalent binding of diclofenac (DCF) (A), 4'-hydroxydiclofenac (B) and 5-hydroxydiclofenac (C) in livers isolated from rats treated with lipopolysaccharide (LPS), DCF or LPS/DCF. For LPS-treated groups, “–”, “+” and “++” represent intravenous treatment with saline, 1 μg/kg of LPS, and 10 μg/kg of LPS, respectively and for DCF-treated groups, “+” represents intraperitoneal treatment with 20 mg/kg of DCF. Data are shown as fold changes to treatment with DCF alone and as means ± S.D. (n = 4). Asterisks indicate statistically significant difference (* P < 0.05, vs treatment with DCF alone).
was associated with a decrease in hepatic GSH level. Further- more, we revealed that adducts of 5-OH-DCF to rat hepatic tissues were significantly increased by co-treatment with LPS and DCF and that this increase in covalent binding of 5-OH-DCF to hepatic tissues amplifies the potential to cause an immune response (Naisbitt et al., 2004) have proposed that both 4'-OH-DCF and acyl glucuronide provide an antigenic determinant for the immune response. Evans et al. (2004) have proposed that both in vitro human liver microsomes and rat in vivo covalent binding yields of < 50 pmol/mg protein are desirable as a rationale for compound evaluation, but it would mean that the drugs exhibiting covalent binding yields higher than 50 pmol/mg protein are all problematic, suggesting that an increase in covalent binding of reactive metabolites could be a risk factor of IDT. These findings indicate that an increase in covalent binding of 5-OH-DCF to hepatic tissues amplifies the potential to cause an immune reaction to the 5-OH-DCF conjugate as an antigen and results in the onset of DCF-induced IDT.

In summary, we demonstrated that co-treatment of rats with LPS and DCF at a non-hepatotoxic dose induced liver injury associated with increased plasma ALT and AST activities. Furthermore, we revealed that adducts of 5-OH-DCF to rat hepatic tissues were significantly increased by co-treatment with LPS and DCF and that this increase was associated with a decrease in hepatic GSH level. These results indicate that covalent binding of 5-OH-DCF to hepatic tissues may play an important role in the onset of the DCF-induced IDT, especially under decreased GSH conditions due to inflammation or oxidative stress.

ACKNOWLEDGMENTS

The authors thank Mr. Tetsuaki Takahashi, Ms. Chizuru Nishiyama and Mr. Ayaki Murayama for their technical help in animal experiments. We also thank Ms. Miyuki Tsuda, Ms. Sachika Suzuki and Mr. Yoshiyuki Motokawa for their technical assistance.

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Effect of LPS on diclofenac reactive metabolites in rats

4'-OH-DCF and acetyl glucuronide provide an antigenic determinant for the immune response (Naisbitt et al., 2007). In vivo rat hepatic tissues and saline control groups. Data are shown as means ± S.D. (n = 4-6). Asterisks indicate statistically significant difference (* P < 0.05, vs control).

**Fig. 8.** Glutathione (GSH) concentrations in livers isolated from rats treated with lipopolysaccharide (LPS), diclofenac (DCF) or LPS/DCF. For LPS-treated groups, “−” and “++” represent intravenous treatment with saline and 10 μg/kg of LPS, respectively and for DCF-treated groups, “−” and “+” represent intraperitoneal treatment with saline and 20 mg/kg of DCF, respectively. The saline-treated group served as a control group. Data are shown as means ± S.D. (n = 4-6). Asterisks indicate statistically significant difference (* P < 0.05, vs control).


