FORMATION OF A NOVEL QUINONE EPOXIDE METABOLITE OF TROGLITAZONE WITH CYTOTOXIC TO HepG2 CELLS

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ABSTRACT:

Troglitazone, an oral antidiabetic drug, was reported to cause adverse hepatic effects in certain individuals, leading to its withdrawal from the market. After incubation of troglitazone (100 μM) with the human hepatoma cell line, HepG2 cells, and human primary hepatocytes for 48 to 72 h, an unknown peak was detected in the cell culture. The formation of this peak from troglitazone (100 μM) was also catalyzed by expressed CYP3A4, and further HPLC analysis revealed that there were three metabolites (metabolite A, B, and C) in the peak. The major metabolite, metabolite C (M-C), was identified as an epoxide of a quinone metabolite of troglitazone by comparison with a synthetic authentic standard using tandem mass spectrometry, 1H NMR, and 13C NMR analyses. The other two metabolites (M-A and M-B) were stereoisomers with the same molecular weight as M-C, probably produced from M-C by intramolecular rearrangement at the epoxide moiety. M-C showed a weak cytotoxicity in HepG2 cells at low concentrations, as assessed by the crystal violet-staining assay. Since epoxides are generally regarded as the chemically reactive species, M-C may play a role in idiosyncrasy of troglitazone hepatotoxicity via individual differences either in the formation or degradation of this metabolite.

Troglitazone is an antidiabetic agent that increases the insulin sensitivity of target tissues in noninsulin-dependent diabetes mellitus (Nolan et al., 1994; Fujiwara et al., 1998). Although troglitazone-associated hepatitis is thought to be rare, there are clinical reports of severe hepatic reactions associated with the use of troglitazone. During clinical trials of troglitazone, 1.9% of patients have experienced an increase in alanine aminotransferase levels greater than 3 times the upper limit of the normal range (Watkins and Whitcomb, 1998). Hepatic injury was reported to occur after short- and long-term troglitazone treatment in the United States (PDR, 2000) and after troglitazone treatment for a period longer than 4 weeks in Japan (Kuramoto et al., 1998). The hepatic toxicity of troglitazone was not observed in any experimental animals tested, including monkeys, which showed similar metabolite profiles as humans (SBA, 1997). Although the mechanism by which troglitazone causes liver dysfunction in certain individuals is not yet clear, it is thought to be idiosyncratic. However, there is no clear statement whether a metabolic idiosyncrasy or immunological idiosyncrasy causes this side effect.

Troglitazone has been reported to be mainly metabolized to sulfate (M-1) and glucuronide (M-2) conjugates (Izumi et al., 1997a,b; Kawai et al., 1998). The oxidized metabolite, a quinone-type metabolite (M-3), was found in humans and monkeys (PDR, 2000). M-1 and M-3 accounts for about 70 and 10% of the metabolites detected in human plasma, respectively (Loi et al., 1997, 1999). Troglitazone has been reported to be a potential inducer of CYP3A4 (PDR, 2000). We reported that troglitazone was oxidized to M-3 by CYP2C8 and CYP3A4 in human liver microsomes (Yamazaki et al., 1999). In general, quinone-type metabolites are considered to be active intermediates in hepatic toxicity induced by such drugs as acetaminophen and other drugs (Pumford and Halmes, 1997; Bort et al., 1999). Recently, it was reported that reactive metabolites are trapped as GSH1 conjugates derived from troglitazone and M-3 in the presence of human liver microsomes (Kassahun et al., 2001). However, the assumed reactive metabolites were not isolated, and therefore, no toxicological information is available.

In the present study, we identified potentially reactive, novel metabolites of troglitazone by MS/MS and NMR analyses. In the course of our studies with human hepatocytes, we found that the formation of the novel metabolites of troglitazone is catalyzed by CYP3A4 in vitro. This finding raised the possibility that troglitazone would undergo P450-mediated conversion to the active metabolite(s) and that it may play a role in troglitazone-induced hepatotoxicity rather than troglitazone itself. Furthermore, we investigated the cytotoxic effects of troglitazone and its novel metabolites in HepG2 cells.

Materials and Methods

Chemicals. Troglitazone and its metabolites were synthesized by Sankyo (Tokyo, Japan). Synthetic standard substances A, B, and C, corresponding to biologically generated metabolites M-A, M-B, and M-C, respectively, were prepared from M-3 by reaction with 2 equimolar 80% m-chloroperbenzoic acid.
in dichloromethane for 2 days at room temperature and following silica gel chromatography. The chemical structures of standard substances A, B, and C were confirmed by MS and NMR analyses, as described in the text. Rifampicin was obtained from Wako Pure Chemicals (Osaka, Japan). Lanford’s medium was provided by Daiichi Pure Chemicals (Tokyo, Japan). All other reagents used in this study were of analytical grade.

**Enzyme Preparation.** Rabbit NPR (Guengerich et al., 1981) and human h$_3$ (Shimada et al., 1986) were purified from liver microsomes by the methods described. Recombinant CYP3A4 was purified from *Escherichia coli* membranes, as described elsewhere (Gillam et al., 1993).

**Cell Culture.** HepG2 cells were obtained from Riken Gene Bank (Tsukuba, Japan). The cells were cultured in Dulbecco’s modified Eagle’s medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (Invitrogen, Paisley, UK) in a humidified atmosphere in 5% CO$_2$ at 37°C. Cells were seeded on 12-well plates at a cell density of 1 × 10$^5$ cells/well. Human hepatocytes were obtained from Charles River Japan (Yokohama, Japan). The viability of cells was measured by a trypan blue exclusion test. Viable hepatocytes were plated in Lanford’s medium on collagen-coated 24-well plates at a cell density of 5 × 10$^4$ cells/well. Unattached cells were removed by changing the medium 24 h after seeding. The chemicals dissolved in dimethyl sulfoxide were added at a final concentration of the solvent not higher than 0.3% in culture medium.

**Oxidative Metabolism of Troglitazone.** Troglitazone (100 μM) was added to HepG2 cells and human hepatocytes and incubated for 48 h and 72 h, respectively. When rifampicin was used, human hepatocytes were coincubated with rifampicin (50 μM) and troglitazone. The reaction was terminated by adding ice-cold ethanol to the samples (1:1, v/v). The standard incubation mixtures in a cell-free system (final volume of 0.2 ml) contained purified CYP3A4 (63 nM), NPR (125 nM), and h$_3$ (63 nM), phospholipid mixture (0.02 mg/ml), cholate (0.25 mM), 100 mM Tris-HCl buffer, pH 7.4, 1 mM NADPH, and troglitazone (100 μM). Incubation was carried out at 37°C for 30 min and terminated by adding 0.2 ml of ice-cold ethanol. The resulting precipitate was removed by centrifugation (2,500 rpm, 10 min).

The metabolites in the supernatant were analyzed by HPLC with a C$_{18}$ (5 μm) analytical column (4.6 × 150 mm; YMC-Pack A-302; YMC Co., Kyoto, Japan). The elution was conducted with a mobile phase containing 42% acetonitrile/0.05% phosphoric acid (v/v) at a flow rate of 2.0 ml/min, and detection was by UV absorbance at 230 nm (system 1; Kawai et al., 1998; Yamazaki et al., 1999). In the case of further separation of new metabolites of troglitazone, another column (6.0 × 150 mm; YMC-Pack A-312) was used with a mobile phase containing 50% acetonitrile/0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min (system 2).

**Identification of Troglitazone Metabolites.** Three metabolites were identified by comparison with the chemically synthesized standard substances A, B, and C using a Q-TOF hybrid-type MS/MS spectrometer (Micromass, Wythenshawe, Manchester, UK) under electrospray ionization (ESI) conditions (Q-TOF). The operation conditions used were: capillary, 3.3 kV; cone, 50 V; source block temperature, 120°C; desolvation gas temperature, 300°C; collision gas, Xe; collision energy, 15 eV; and analyzer vacuum, 4.5 to 5.0 × 10$^{-5}$ Torr. The LC condition in the LC-MS/MS analysis was system 2, previously described above. The samples were injected to an LC system, and the outflow from the column was split by a tee splitter (100 μl/min) for Q-TOF/MS analysis.

The synthesized standard substances were dissolved in a total 0.5 ml of CDCl$_3$ (ISOTEC, Inc., Miamisburg, OH; 99.8% D) or DMSO-d$_6$ (dimethyl-sulfoxide; ISOTEC, Inc.; 99.96% D), respectively. One-dimensional $^1$H-complete-decoupled and DEPT135 spectra were recorded on a 500 MHz spectrometer (JNM-A500; JEOL, Tokyo, Japan), whereas one-dimensional $^1$H, two-dimensional $^1$H-$^1$H, and $^1$H-$^1$C spectra were recorded on a 600 MHz spectrometer (DMX600, Bruker, Newark, DE). $^1$H and $^{13}$C chemical shifts were referred to the CDCl$_3$ signal at 7.20 and 77.7 ppm, respectively.

**Cytotoxicity Assay.** Cell viability was assessed by the crystal violet-staining assay, according to Nakagawa et al. (1996). HepG2 cells were seeded onto 12-well plates at a cell density of 1 × 10$^5$ cells/well. Cells were incubated with the thiazolidinedione chemicals for 48 h. After the incubation, the plates were washed with phosphate-buffered saline and fixed with 3.7% formaldehyde. The cells were stained with 0.2% crystal violet. The absorbance of the extracts with 2% sodium dodecyl sulfate at 620 nm was measured. The cell viability was calculated by comparison with the absorbance of cells incubated without the chemicals.

**Data Analysis.** Nonlinear regression analysis by the computer program KaleidaGraph (Synergy Software, Reading, PA) was used to determine LC$_{50}$, LC$_{50}$, and the Hill slope using the equation % L$_{50}$ = 100 − (LC$_{50}$ × $S^n$)/(LC$_{50}$ + S$^n$). LC$_{50}$ is the substrate concentration showing the half-maximal lethal toxicity, n is the slope factor, and L$_{max}$ is the maximal lethal toxicity. Estimates of variance (S.E., denoted by ±) are presented from the analysis of individual sets of data.

**Results**

**Metabolism of Troglitazone.** The metabolism of troglitazone was investigated using HepG2 cells and human primary hepatocytes. Typical chromatograms are shown in Fig. 1. After incubation of troglitazone (100 μM) with HepG2 cells and human hepatocytes, the forma-
tion of several metabolites was observed in the culture medium. Peaks with retention times of 7.8, 10.0, and 22.7 min were assigned to M-1, M-3, and troglitazone, respectively, by comparison with authentic standard substances. The nonenzymatic conversion of troglitazone to M-3 was less than 5% of the formation by cells. The other peak at 7.5 min (designated as an unknown peak) was present both in the culture medium of the HepG2 cells and human hepatocytes. The formation of this unknown peak was increased by cotreatment of human hepatocytes with rifampicin (50 μM), a CYP3A4 inducer. This unknown peak was not observed in the absence of the cells.

The formation of M-3 and the unknown peak at 7.5 min was also observed after incubation of troglitazone (100 μM) with the reconstituted systems containing CYP3A4 (Fig. 2A). The major metabolite detected was the unknown peak at 7.5 min under these conditions. Incubation of M-3 instead of troglitazone as a substrate failed to produce the unknown peak. The unknown peak at 7.5 min was further separated into three peaks, designated as peak A (M-A), peak B (M-B), and peak C (M-C) under system 2 (Fig. 2B).

**Structural Confirmation of the Synthetic Authentic Standard**

**Substances A, B, and C.** Three authentic standard substances, A, B, and C, had the same molecular weight of 473, demonstrating an introduction of one oxygen atom to the quinone metabolite of troglitazone, M-3 (See Fig. 3 for standard substance C and M-C; data not shown for substances A and B). In Fig. 4, the $^1$H NMR spectra of A, B, C, and M-3 are shown. In the spectrum of C, the chemical shifts of the protons of the methyl group at the 14-position were unchanged compared with those of the corresponding methyl group of M-3, showing that the 14-methyl group is not chemically modified in C. On the other hand, the chemical shifts of the 17- and 22-carbons of C were shifted to a higher field than those of M-3, with other chemical shifts being almost unchanged. These results demonstrated that C has an epoxide moiety at the 17- and 22-carbons. The observation of long-range couplings in the heteronuclear multiple-bond correlation spectroscopy spectrum was consistent with this conclusion (data not shown).

In a similar manner as C, a higher field shift of the $^1$H signals of
22-methyl protons and the $^{13}$C signals of 17- and 22-carbons were observed in A and B compared with those in M-3. The higher field shift of the $^{13}$C signals of the 22-carbons in A and B was less extent than that observed in C, indicating that these carbons in A and B are normal clincher carbons having a hydroxyl group. The spiro[4,5]decadion ring units of A and B were assigned based on the normal clincher carbons having a hydroxyl group. The 22-methyl protons and the 13 C signals of 17- and 22-carbons were observed in A and B compared with those in M-3. The higher field shift of the 13 C signals of the 22-carbons in A and B was less extent than that observed in C, indicating that these carbons in A and B are normal clincher carbons having a hydroxyl group. The spiro[4,5]decadion ring units of A and B were assigned based on the normal clincher carbons having a hydroxyl group.

**Fig. 4.** $^1$H NMR spectra of the authentic standards A, B, C, and M-3 in CDCl$_3$ and deduced structures.

Identification of Troglitazone Metabolites. The unknown peak, M-A, M-B, and M-C generated by CYP3A4 were analyzed by MS and MS/MS using various ionization modes. The ESI-MS spectra of M-A, M-B, and M-C generated by CYP3A4 were analyzed by MS and MS/MS using various ionization modes. The ESI-MS spectra of the authentic standards A, B, C, and M-3 in CDCl$_3$ and deduced structures.

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Cytochrome P450 enzymes catalyze the oxidation of a broad spectrum of endobiotic and xenobiotic substrates. The resulting hydroxylated metabolites are more hydrophilic, facilitating their renal and biliary excretion. Drug oxidation generally leads to termination of the pharmacological potency, but examples exist of drug oxidation constituting a pharmacological or toxicological bioactivation event. Epoxidation is one of the most important metabolic activation pathways resulting in toxic effects. Epoxides are chemically unstable or reactive and have been reported to bind covalently to the nucleic acids or proteins of tissue (Kemper et al., 1995). This effect also may lead to such consequences as the production of autoantibodies, inflammation, and cancer. On the other hand, epoxides are eliminated by glutathione S-transferase or epoxide hydrolase in rodents and humans. The depletion of GSH or a decrease of glutathione will bind to DNA or proteins is determined by the rate of activation of the epoxide and the rates of hydroxylation and GSH conjugation.

**Discussion**

Cytochrome P450 enzymes catalyze the oxidation of a broad spectrum of endobiotic and xenobiotic substrates. The resulting hydroxylated metabolites are more hydrophilic, facilitating their renal and biliary excretion. Drug oxidation generally leads to termination of the pharmacological potency, but examples exist of drug oxidation constituting a pharmacological or toxicological bioactivation event. Epoxidation is one of the most important metabolic activation pathways resulting in toxic effects. Epoxides are chemically unstable or reactive and have been reported to bind covalently to the nucleic acids or proteins of tissue (Kemper et al., 1995). This effect also may lead to such consequences as the production of autoantibodies, inflammation, and cancer. On the other hand, epoxides are eliminated by glutathione S-transferase or epoxide hydrolase in rodents and humans. The depletion of GSH or a decrease of glutathione will bind to DNA or proteins is determined by the rate of activation of the epoxide and the rates of hydroxylation and GSH conjugation.

Troglitazone is metabolized to sulfate (M-1), glucuronide (M-2), and a quinone-type metabolite (M-3) in both humans and experimental animals (Izumi et al., 1997a,b; Kawai et al., 1998; PDR, 2000). The glucuronide of the hydroquinone form of troglitazone has been isolated from the bile and urine in monkeys and marmosets, which show similar metabolic profiles as humans (Kawai et al., 1998). Recently, P450-dependent reactive metabolites of troglitazone and M-3 have been trapped as the conjugates of GSH in vitro and in vivo (Kassahun et al., 2001). The biotransformation of these conjugates has been assumed to involve quinone methide formation and thiazolidinedione ring scission.

In the present study, we found an epoxide-form metabolite of troglitazone, not reported previously, as the major component (M-C) of a novel peak in the culture medium of human primary hepatocytes (Fig. 1), used as an in vitro device to extrapolate the in vivo situation. The formation of M-C was increased in the cells coinubated with rifampicin, an inducer of CYP3A4 in humans (Fig. 1C), and was dependent on NADPH in the cell-free systems containing CYP3A4 (data not shown). These results indicate that M-C formation is catal-
alyzed by P450, especially by CYP3A4. The peak was also observed previously as a very small peak compared with M-3 after the incubation of troglitazone with human liver microsomes or with cDNA-expressing human CYP2C8 and CYP3A4, although we did not attempt to isolate this peak (Yamazaki et al., 1999). Identification of these novel metabolites was done in the present study by MS/MS (Fig. 3) and 1H NMR (Fig. 4) or 13C NMR (Fig. 5), using synthetic authentic standards. In addition to the identification of M-C as the quinone epoxide, M-A and M-B were found to be the products probably produced by intramolecular rearrangement of M-C, with the epoxide group being attacked by the hydroxyl group at the 14-position (Figs. 2 and 6). Although the epoxide structure of quinone is rather unusual, the photooxidation product of troglitazone, having exactly the same structure as M-C, has been reported (Fu et al., 1996). Tocopherol has been also shown to undergo epoxidation at the benzooquinone moiety (Clough et al., 1979). The presence of M-A and M-B as the chemically derived substances from M-C also supports the chemical structure of M-C. Since the epoxides are generally supposed to be chemically reactive, we examined the cytotoxicity of M-C in comparison with M-3 and troglitazone in the system using HepG2 cells. Although the use of freshly isolated human hepatocytes is relevant to the in vivo situation, these cells are not readily available, and we used HepG2 cells as the liver cells of human origin. M-C was weakly cytotoxic to HepG2 cells from low concentrations (not less than 12.5 μM) but less cytotoxic than troglitazone at high concentration. This was contrasted with the complete lack of cytotoxicity of the conjugated metabolites M-1 and M-2 (Yamamoto et al., 2002).

The maximum plasma concentrations in the patients taking troglitazone at therapeutic doses of 400 and 600 mg/day have been reported to be 3.6 to 6.3 μM (Loi et al., 1999). Tissue distribution studies in rats have shown that the concentrations of troglitazone within liver tissues are much higher (10- to 12-fold) than those in the plasma (Sahi et al., 2000). These findings suggested that the troglitazone levels in human livers would reach such concentrations, allowing M-C formation in vivo. Moreover, troglitazone has been shown to act as an inducer of CYP3A4 that catalyzes its own oxidative metabolism (Sahi et al., 2000). Therefore, treatment of patients with troglitazone for a relatively long period may lead to an increased production of M-C,
and such autoinduction of the metabolism formation may be one of the factors in the etiology of troglitazone-mediated liver injury.

In conclusion, we found a novel epoxide-form metabolite of troglitazone with direct cytotoxic effects on human hepatocytes. The importance of this finding in elucidating the troglitazone hepatotoxicity is unknown. However, since epoxides are generally supposed to be chemically reactive metabolites, they may play a certain role in the idiosyncrasy of troglitazone hepatotoxicity via individual differences, either in the formation or degradation of epoxide-form metabolite in humans.

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References


