てルシフェラーゼ活性の低下が認められたことから、MRE378 が miR-378 により認識され、発現制御に機能的に働いていることが示唆された。また、3'-UTR を含むまたは含まない CYP2E1 安定発現 HEK293 細胞を構築することで、CYP2E1 タンパク質発現量への miR-378 の影響を検討した。3'-UTR を含む CYP2E1 安定発現細胞株においてのみ、pre-miR-378 導入による CYP2E1 タンパク質発現量の低下が認められた。このことからも、MRE378 を含む 3'-UTR の存在が miR-378 による CYP2E1 の発現抑制に重要な役割を果たしていることが示された。次に、miR-378 による CYP2E1 発現調節が実際にヒト肝臓中でも起きている事象であるか、25 検体のヒト個人肝における CYP2E1 mRNA とタンパク質発現量および酵素活性、mature miR-378 発現量の相関関係を調べることで評価した。CYP2E1 mRNA 発現量とタンパク質発現量との間には正の相関関係が認められず、また、miR-378 発現量と CYP2E1 タンパク質発現量および CYP2E1 翻訳効率との間に有意な逆相関が認められたことから、miR-378 による翻訳抑制がヒト肝における CYP2E1 の常在的な発現に寄与していることが示唆された。未解明であった CYP2E1 の転写後調節機構に対して、miRNA による翻訳抑制機構を新たに提唱することが出来た。miR-378 による CYP2E1 の発現制御は、ヒト肝における CYP2E1 発現量の個人差を引き起こす要因の 1 つとして考えられる。

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E. 健康危険情報

該当なし。

F. 研究発表

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G. 知的財産権の出願・登録

該当なし。

III. 研究成果の刊行に関する一覧表

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
Tsuyoshi Yokoi	Troglitazone	Jack Uetrecht	Adverse Drug Reaction	Springer-Verlag	Berlin	2010	419-435
横井　毅	薬物代謝と毒性発現	加藤隆一 山添　康 横井　毅	薬物代謝学第３版	東京化学同人	東京	2010	43-68, 182-192

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横井　毅	第II相代謝の評価と創薬	日本薬理学雑誌	134	334-337	2010

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Takuya Mohri, Miki Nakajima, Tatsuki Fukami, Masataka Takamiya, Yasuhiro Aoki and Tsuyoshi Yokoi	Human CYP2E1 is regulated by miR-378.	***Biochemical Pharmacology***	79	1045-1052	2010
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Hiroko Hosomi, Tatsuki Fukami, Atsushi Iwamura, Miki Nakajima and Tsuyoshi Yokoi.	Development of a highly sensitive cytotoxicity assay system for CYP3A4-mediated metabolic activation.	***Drug Metabolism and Disposition***		in press	2011

VI. 研究成果の刊行物・別刷

Troglitazone

Tsuyoshi Yokoi

Contents

Abstract Troglitazone was the first thiazolidinedione antidiabetic agent approved for clinical use in 1997, but it was withdrawn from the market in 2000 due to serious idiosyncratic hepatotoxicity. Troglitazone contains the structure of a unique chroman ring of vitamin E, and this structure has the potential to undergo metabolic biotransformation to form quinone metabolites, phenoxy radical intermediate, and epoxide species. Although troglitazone has been shown to induce apoptosis in various hepatic and nonhepatic cells, the involvement of reactive metabolites in the troglitazone cytotoxicity is controversial. Numerous toxicological tests, both *in vivo* and *in vitro*, have been used to try to predict the toxicity, but no direct mechanism has been demonstrated that can explain the hapatotoxicity that occurred in some individuals. This chapter summarizes the proposed mechanisms of troglitazone hepatotoxicity based *in vivo* and in vitro studies. Many factors have been proposed to contribute to the mechanism underlying this idiosyncratic toxicity.

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J. Uetrecht (ed.), *Adverse Drug Reactions*, Handbook of Experimental pharmacology 196, 419
DOI 10.1007/978-3-642-00663-0_14,

Keywords Troglitazone · Hepatotoxicity · Active metabolites · CYP3A · Hypersensitivity

1 Introduction

Thiazolidinediones (Fig. 1) are a class of oral antidiabetic agents, which are a synthetic ligand for the peroxisome proliferator-activated receptor γ (PPARγ) (Lehmann et al. 1995). Troglitazone (Rezulin®, (±)-5-[[4-[(3,4-dihydro-6-hydroxy-2,5,7,8-tretramethyl-2H-1-benzopyran-2-yl)methoxy]phenyl]methyl]-2,4-thiazolidinedione) was the first thiazolidinedione antidiabetic agent approved for clinical use by the US in 1997. Troglitazone lowers blood glucose levels through increased glucose uptake by skeletal muscle, decreased hepatic glucose production, and increased insulin sensitivity of the target tissue in animal models of metabolic impairment (Ciaraldi et al. 1990; Fujiwara et al. 1995). These pharmacological effects are exerted through PPARγ-dependent transcription of genes involved in glucose and lipid metabolism and energy homeostasis (Lehmann et al. 1995; Saltiel and Olefsky 1996; Spiegelman 1998). Based on the pharmacological advantages and the apparent absence of serious toxic effects, troglitazone was thought likely to become a promising treatment for type II diabetes mellitus in patients with insulin resistance.

In the combined North American clinical trials, elevations of serum alanine aminotransferase (ALT) more than three times the upper limit of normal were observed in 48 out of 2,510 patients (1.9%) treated with troglitazone as compared

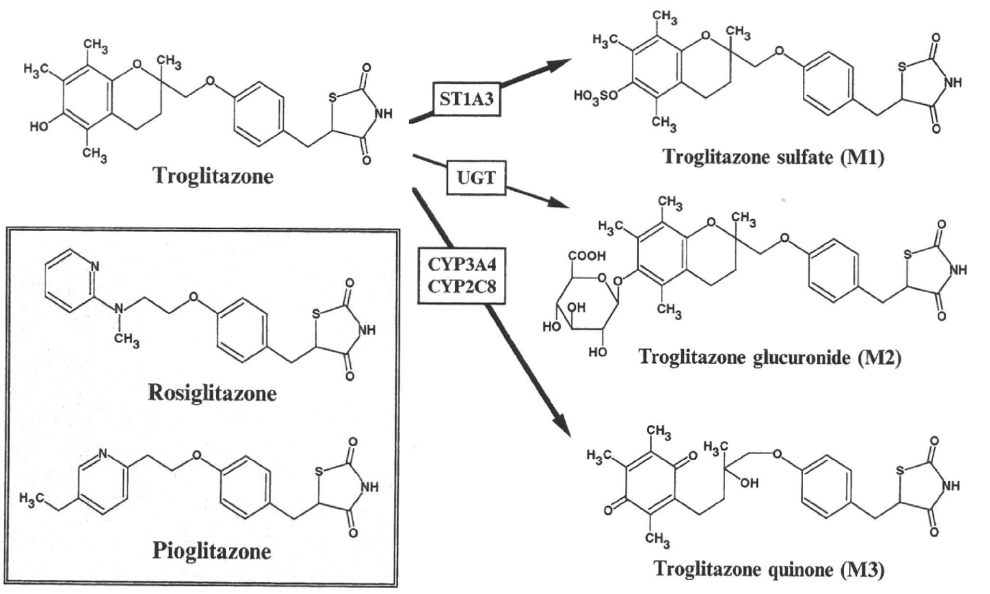

Fig. 1 Structures of thiazolidinediones and pathways of troglitazone metabolism to relatively stable metabolites

to 0.6% in patients who received placebo. Two patients were confirmed to have suffered serious hepatocellular injury from an idiosyncratic drug reaction (Watkins and Whitcomb 1998). Meanwhile, troglitazone was concomitantly reported to be associated with idiosyncratic hepatotoxicity with some patients showing severe or fatal liver damage (Gitlin et al. 1998; Neuschwander-Tetri et al. 1998; Shibuya et al. 1998). Consequently, it was withdrawn from the market in the US and Japan in March 2000. The hepatotoxic effects of troglitazone were not predicted in conventional experimental animals (Watanabe et al. 1999) or in cynomolgus monkeys (300–1,200 mg/kg/day for 52 weeks), a primate model having similar metabolic profiles to humans (Rothwell et al. 2002). Two other thiazolidinediones which are now on the market, rosiglitazone and pioglitazone, were introduced in 1999, and they appear not to exhibit the hepatotoxic effects of troglitazone (Freid et al. 2000; Isley and Oki 2000; Lebovitz et al. 2002), although an association with hepatotoxicity has been reported in very rare instances (Nagasaki et al. 2002). It should also be noted that the clinical dosage regimen for improvement of the fasting glucose level differs among these thiazolidinediones. The recommended dose for troglitazone was 200–600 mg/day, for rosiglitazone 4–8 mg/day, and for pioglitazone 15–45 mg/day (Hanefeld 2001; Loi et al. 1999; PDR 1999, 2005a, b). The Cmax and AUC of troglitazone are 0.90–2.82 mg ml^{-1} and 7.4-22.1 mg-h ml^{-1}, respectively, whereas those of rosiglitazone are 0.076–0.598 mg ml^{-1} and 0.358–2.971 mg-h ml^{-1}, respectively. The plasma elimination half-life and biliary excretion of troglitazone are 16–34 h and 85%, respectively, and those of rosiglitazone are 3–4 h and 23%, respectively. Although the dosage for sufficient pharmacological efficacy could be related to the hepatotoxic potential, the mechanism of troglitazone toxicity is still controversial. Numerous toxicological tests, both *in vivo* and in vitro, have been attempted, but no direct mechanism has been successfully demonstrated that can explain the hepatotoxicity that occurred in some individuals.

Troglitazone represents a model of an idiosyncratic drug reaction that led to withdrawal from the market and to attempts to understand the mechanisms of such adverse drug reactions. This review summarizes the proposed molecular mechanisms of troglitazone hepatotoxicity based on both *in vivo* and in vitro studies. However, so far, there is no direct evidence indicating the precise mechanism of the toxicity. Many factors have been proposed to contribute to this idiosyncratic toxicity.

2 Metabolism of Troglitazone into Stable Metabolites

The bioavailability of troglitazone is 40–50%, which can be affected by food and other factors (Loi et al. 1999). The plasma protein binding is more than 99%, and the distribution into red blood cells is low (Kawai et al. 1997). In humans, there is no evidence that troglitazone accumulates in the liver. However, troglitazone is absorbed in isolated perfused livers and cultured hepatocytes within minutes, even in the presence of albumin or serum (Preininger et al. 1999; Haskins et al. 2001;

Yamamoto et al. 2001). In humans, troglitazone is predominantly metabolized by three pathways: sulfation, glucuronidation, and oxidation, to form a sulfate conjugate (M1), a glucuronide conjugate (M2), and a quinone metabolite (M3), respectively (Fig. 1). The main metabolite, troglitazone sulfate (M1), is catalyzed by phenol sulfotransferase, ST1A3 (Honma et al. 2002), and accounts for about 70% of the metabolites detected in human plasma (Loi et al. 1999), exceeding that of the parent drug. Troglitazone sulfate undergoes enterohepatic circulation after biliary excretion resulting in a long half-life *in vivo* in humans (Kawai et al. 1998), which may be involved in cholestatic liver injury through inhibition of bile acid transport as described below.

A relatively minor metabolite, troglitazone glucuronide (M2), is catalyzed by UGT (Yoshigae et al. 2000). The glucuronidation of troglitazone in human intestine is threefold higher than that in human liver. In the liver, the reaction is likely mediated by UGT1A1, while in the intestine it is mediated by UGT1A8 and UGT1A10 (Watanabe et al. 2002). Furthermore, in enterocytes, it may also be converted to glucuronide by UGTs such as UGT1A8 and UGT1A10.

In human liver, CYP3A4, CYP2C8, and CYP2C19 mainly catalyze troglitazone to a quinone-type metabolite (M3). The chroman ring of vitamin E can be oxidized to a quinone. Kinetic analysis of the troglitazone oxidation (M3 formation) by recombinant P450 enzymes showed that CYP3A4, CYP2C8, and CYP2C19 had relative clearance values of 0.4, 1.6, and 0.9 ml $min^{-1}nmol^{-1}$ P450, respectively (Yamazaki et al. 1999). Considering the relative P450 enzyme contents in human liver, CYP3A4 may be expected to play a major role in the formation of a quinone-type metabolite from troglitazone even at a low concentration. The quinone metabolite M3 is relatively stable and exhibited weaker cytotoxicity than troglitazone (Yamamoto et al. 2001). In addition, troglitazone has been shown to induce CYP3A in human and rat hepatocytes, which stimulates the formation of the quinone metabolites (Ramachandran et al. 1999; Sahi et al. 2000). Therefore, the large interindividual variability of CYP3A4 activities in human liver may be related to the risk of troglitazone-induced hepatotoxicity.

3 Reactive Metabolites and Cytotoxicity

Differing from other thiazolidinediones, troglitazone contains a chroman ring of the vitamin E moiety. This structure accounts for the effective antioxidant property of troglitazone and suggests an advantage in preventing diabetic vascular complications in addition to its hypoglycemic and hypolipidimic effects (Inoue et al. 1997). This structure, however, has the potential to undergo metabolic activation to form the troglitazone quinone metabolite (M3). As mentioned above, although the quinone metabolite M3 is relatively stable, by the action of CYP3As, troglitazone yields several reactive intermediates (Kassahun et al. 2001; Tettey et al. 2001; He et al. 2001) (Fig. 2). The formation of an epoxide of troglitazone quinone was also identified in vitro in humans (Yamamoto et al. 2002) and is likely to be a potent

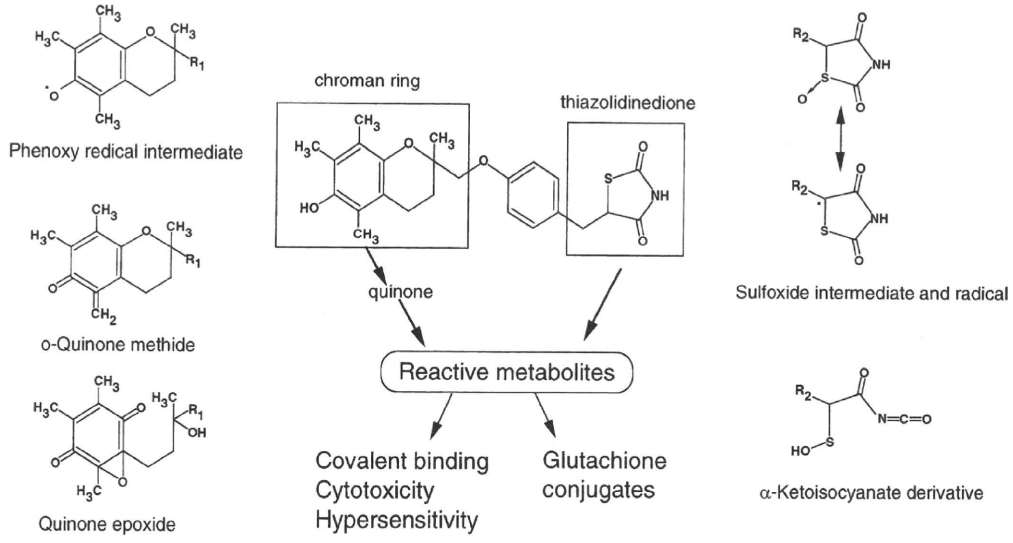

Fig. 2 Reactive metabolites of troglitazone catalyzed by CYP3A potentially leading to toxicity

electrophile. Although the troglitazone quinone does not react directly with GSH, it can be further metabolized to an *o*-quinone methide or undergo ring opening to produce additional highly electrophilic intermediates (Kassahun et al. 2001). Such electrophilic intermediates are toxicologically active, which can result in acute cytotoxicity and immunotoxicity as well as carcinogenesis (Bolton et al. 2000).

Cytotoxicity assays of troglitazone and its metabolites were performed in various types of cells, such as HepG2 cells and hepatocytes from human and experimental animals. The maximum plasma concentrations in patients taking troglitazone at a dose of 600 mg/day only reached about 2.82 μg ml^{-1} or 6.3 μM (Loi et al. 1999). However, a study in rats demonstrated that the concentration of troglitazone in liver tissues was 10- to 12-fold higher than that in the plasma (Sahi et al. 2000). Therefore, the troglitazone levels in human liver might allow the formation of these reactive intermediates, and their accumulation may lead to the hepatotoxicity. In the cytotoxicity assay, the estimated IC50 values of troglitazone and the quinone metabolite, M3, were 34 and 66 μM, respectively, in HepG2 cells incubated in a 5% FBS-containing culture medium (Yamamoto et al. 2001). These reports suggested that the troglitazone levels in human livers could reach such concentrations, which may cause the observed cytotoxicity *in vivo*.

The reactive metabolite(s) covalently binds to cellular macromolecules, but the role of the protein adduct on troglitazone-induced cytotoxicity is still controversial. Using cryopreserved human hepatocytes from 27 individuals, none of the individual phase I or II enzyme activities correlated with the EC50 values of troglitazone cytotoxicity (Hewitt et al. 2002). However, a combination of high CYP3A4 and UGT activities was associated with low toxicity while low CYP3A4 with high ST activity was associated with higher toxicity, which suggested that troglitazone sulfate might act as direct toxicant, and CYP3A4 and UGT were involved in detoxification (Hewitt et al. 2002). On the other hand, chemical inhibitors of drug metabolizing enzymes were employed to elucidate their involvement in the

cytotoxicity to HepG2 cells. Ketoconazole (an inhibitor of CYP3A4), quercetin (an inhibitor of CP2C8), and DCNP (2,4-dichloro-4-nitrophenol, an inhibitor of sulfation) did not successfully attenuate the cytotoxicity in HepG2 cells (Yamamoto et al. 2001). However, inhibition of troglitazone sulfation by DCNP and pentachlorophenol resulted in aggravation of cytotoxicity in human hepatocytes (Kostrubsky et al. 2000), indicating a result opposite to that of Hewitt et al. (2002). The use of cultured cell lines in cytotoxicity assays requires careful interpretation because the activities of drug metabolizing enzymes in such cells are very low. However, Vignati et al. (2005) reported that HepG2 cells, together with microsomes expressing human CYPs or HepG2 cells transfected with CYP3A4, were able to metabolize troglitazone resulting in increased cytotoxicity. Established cell lines expressing the same level of drug metabolizing enzymes as those in human liver would be useful for troglitazone-induced cytotoxicity assays.

4 Biomarkers of Susceptibility to Troglitazone Hepatotoxicity

As mentioned above, troglitazone can undergo metabolic biotransformation by CYP3A4 to form quinone and epoxide metabolites (Izumi et al. 1997a, b; Kawai et al. 1998; Loi et al. 1999; Yamamoto et al. 2002). Quinones are well-established cytotoxic agents and can produce toxicity by redox cycling with molecular oxygen to produce superoxide anion radical and subsequent oxidative stress (Schultz et al. 1996; Bolton et al. 2000). Quinones can also react readily with sulfur nucleophiles such as glutathione (GSH) or cysteine residues on proteins (Bolton et al. 2000). However, little information is available about enzymatic detoxification of these reactive metabolites. The toxic effects of troglitazone have been thought to be mediated by the depletion of GSH, covalent binding to cellular macromolecules, or oxidative stress. In cryopreserved human hepatocytes, large variations in the sensitivity to troglitazone were observed, and sensitive donors were demonstrated to form significantly lower amounts of GSH conjugates and glucuronides than resistant donors (Kostrubsky et al. 2000; Prabhu et al. 2002). It is known that GSH conjugation is catalyzed by the action of glutathione *S*-transferase (GST). A study in rats has shown that GSH adducts of troglitazone are formed and the reaction is enhanced by CYP3As (Tettey et al. 2001). An epoxide of troglitazone quinone catalyzed by CYP3A4 might also be eliminated by GSTs and epoxide hydrolase (Yamamoto et al. 2002). These findings indicate an association between metabolic activation by CYP and detoxification by GSTs. In a key report concerning this aspect, Watanabe et al. (2003) investigated the genetic factors responsible for troglitazone hepatotoxicity *in vivo* in humans. Among 110 patients prescribed troglitazone, 25 had an abnormal increase in ALT or AST levels to at least nine times or five times the upper limit of the normal range, respectively, while 85 control patients showed no significant increase in ALT levels during more than 6 months of treatment. Interestingly, they found that this abnormal elevation of liver enzymes caused by troglitazone treatment was highly associated with the double

null genotype of *GSTM1* and *GSTT1* (odds ratio, 3.692; 95% confidence interval, 1.354–10.066; $P=0.008$) (Watanabe et al. 2003). A similar association study regarding hepatotoxicity observed in patients treated with tacrine, a drug used for Alzheimer's disease, was reported by Simon et al. (2000). Thus, interindividual differences in detoxification ability appears to contribute to the susceptibility and individual risk for troglitazone hepatotoxicity. Taking into consideration the double null genotype of *GSTM1* and *GSTT1* in clinical practice, the risk for hepatotoxicity could theoretically be reduced by half.

Recently, we established a GSH-knockdown rat model for the prediction of human hepatotoxicity (Akai et al. 2007). An adenovirus vector with short hairpin RNA against rat γ-glutamylcysteine synthetase (GCS) heavy chain subunit was constructed and used to knockdown GSH synthesis. This rat model, with an 80% decreased hepatic GSH level, demonstrated a high sensitivity for acetaminophen-induced hepatotoxicity. With the advance of molecular biology, novel animal models will be established and applied to drug development in the near future.

5 Inhibition of Hepatic Drug Transporters by Troglitazone Metabolites

Troglitazone sulfate (M1, the main metabolite) undergoes biliary excretion and accounts for up to 85% of the dose in humans (Loi et al. 1999). In patients with hepatic impairment, troglitazone sulfate was found to accumulate about fourfold in plasma with a threefold increased half-life (Ott et al. 1998; Loi et al. 1999). This metabolite also inhibited the canalicular bile salt export pump (Bsep), organic anion transporting polypeptide (OATP) transporters as well as drug transporters, suggesting it contributes to the hepatotoxicity.

Troglitazone sulfate inhibits the ATP-dependent taurocholate transport mediated by Bsep in isolated canalicular rat liver plasma membrane (IC_{50} 0.4–0.6 μM) about ten times more strongly than the parent compound (IC_{50} 3.9 μM) (Funk et al. 2001a, b). When troglitazone sulfate accumulates in hepatocytes at high concentrations, it may disturb the hepatobiliary export of bile acids by the inhibition of Bsep leading to intrahepatic cholestasis in humans. Evidence of cholestasis has also been described in a patient with troglitazone hepatotoxicity (Gitlin et al. 1998).

Troglitazone sulfate was also reported as a substrate of organic anion transporting polypeptide (OATP) transporters with higher affinity to OATP-C (SLC01B1) than OATP8 (SLC01B3). Estrone-3-sulfate was demonstrated to be a potent inhibitor for OATP-C and OATP8 (Nozawa et al. 2004). Both OATP-C and OATP8 are members of the organic anion transporting polypeptides, which are expressed in the basolateral membrane of hepatocytes (Hagenbuch and Meier 2003; Kreb 2006). They play important roles in the hepatic handling of endogenous compounds and xenobiotics. Some types of genetic polymorphisms with functional alterations of OATP-C have been reported, and such alterations may lead to the accumulation of troglitazone sulfate in the liver, resulting in troglitazone-associated hepatotoxicity

(Kreb 2006, Michalski et al. 2002). Taking such information into account, the failure of hepatic excretion of troglitazone sulfate might lead to hepatotoxicity, although troglitazone sulfate itself is pharmacologically inactive and did not exhibit cytotoxicity in human hepatoma cells (Loi et al. 1999; Yamamoto et al. 2001)

Using knockout rats lacking multidrug resistant associated protein-2 (Mrp2), it has been demonstrated that troglitazone glucuronide is a substrate for Mrp2 (Kostrubsky et al. 2001). Therefore, the troglitazone glucuronide formed in enterocytes might be excreted to the intestinal lumen via transporters such as Mrp2 expressed in the brush border membrane. Then, the glucuronide would again be converted to troglitazone by β-glucuronidase and the troglitazone might be reabsorbed. There has been no reported evidence that M2 is responsible for the hepatotoxic effects of troglitazone.

6 Hypersensitivity Reaction Associated with Troglitazone Hepatotoxicity

Idiosyncratic adverse reactions are difficult to study because of their rare occurrence, dose-independence, and lack of reproducibility in experimental animal models. Many idiosyncratic drug reactions have an immunological (hypersensitivity) basis, whereas some are due to a metabolic abnormality of the host (Pohl et al. 1988; Ju and Uetrecht 2002). Idiosyncratic drug-induced hepatitis has been assumed to be mediated by immunogens formed by covalent interaction of a reactive drug metabolite with cellular macromolecules (Park et al. 1998). The bioactivated immunogens may not only lead to an immune response directed against the haptenic epitope and the neoantigen, but also against autoantigenic determinants, which is characterized by the formation of autoantibodies (Pohl et al. 1988). A number of hepatotoxic drugs have been reported to produce autoantibodies. For example, antiprotein disulfide isomerase, antimicrosomal carboxyesterase, anticalreticulin, anti-ERp72, anti-GRP78, anti-GRP94, and anti-CYP2E1in halothane hepatitis (Bourdi et al. 1996; Gut et al. 1993; Kenna et al. 1993; Pumford et al. 1993), anti-CYP2C9 in tienilic acid-induced hepatitis (Homberg et al. 1984; Robin et al. 1996), anti-CYP1A2 in dihydralazine-induced hepatitis (Bourdi et al. 1990), and anti-CYPs in aromatic anticonvulsant-induced hypersensitivities (Leeder et al. 1992) have been reported. However, it is not fully understood whether the autoantibodies are the causes or consequences of hepatotoxicity. Studies to clarify the possible involvement of autoantibodies in drug-induced hepatitis are limited because the appearance of autoantibodies can usually be seen only in humans. We recently reported that aldolase B, which is an enzyme predominantly localized in the liver and kidney (Penhoet et al. 1966), was detected as an autoantigen that reacted with antibodies in the sera from two patients with type II diabetes mellitus and troglitazone-induced liver dysfunction (Maniratanachote et al. 2005b). The titer of antialdolase B remained high for several weeks after stopping troglitazone administration. This finding supported the idea that troglitazone hepatotoxicity may have an immunological basis.

However, autoantibodies to aldolase B were also detected in the sera of patients with chronic hepatitis as well as liver cirrhosis (Brown et al. 1987; Maniratanachote et al. 2005b). There are several reactive metabolites generated by troglitazone (Fig. 2) (Kassahun et al. 2001; Tettey et al. 2001; Yamamoto et al. 2002). Aldolase B, which is an enzyme predominantly localized in the liver (Penhoet et al. 1966), may be one of the target proteins that interact with those reactive species and trigger the immune response. This study suggested that liver dysfunction might cause the appearance of autoantibodies to aldolase B, which may then aggravate the hepatitis. In addition, the antialdolase B titer might indicate the severity of liver dysfunction. Further studies will be needed to clarify the mechanisms of hypersensitivity reactions.

7 Mechanisms of Troglitazone-Induced Hepatotoxicity

Troglitazone has been shown to induce apoptosis in various hepatic (Bae and Song 2003; Tirmenstein et al. 2002; Yamamoto et al. 2001) and nonhepatic (Shiau et al. 2005) cell types depending on the concentration and duration of exposure. Unlike its pharmacological effects, the toxicity of troglitazone seems to be a PPAR γ-independent mechanism, and the higher affinity PPARγ agonists such as rosiglitazone and pioglitazone possess much lower toxic effects (Lehmann et al. 1995; Shiau et al. 2005). In addition, Shiau et al. (2005) demonstrated that a synthetic counterpart of troglitazone, which lacks PPARγ activation activity, was also able to induce apoptosis in cultured cells. A PPARγ-independent mechanism is also possible in human hepatocytes because the expression of PPARγ in normal human liver cells is very low (Green 1995), and rosiglitazone does not induce apoptosis (Toyoda et al. 2001). Troglitazone was shown to inhibit equally the proliferation of both PPAR$\gamma^{-/-}$ and PPAR$\gamma^{+/+}$ mouse embryonic stem cells (Palakurthi et al. 2001).

As mentioned above, M1, M2, and M3 metabolites are relatively stable, and the quinone metabolite, M3, has been suggested to be associated with troglitazone hepatotoxicity in humans (Neuschwander-Tetri et al. 1998). Although these metabolites showed lower toxic effects compared to the parent compound, troglitazone, when mammalian hepatocytes and hepatoma cell lines were treated directly (Tettey et al. 2001; Tirmenstein et al. 2002; Yamamoto et al. 2001, 2002), the possibility that the metabolites are toxic was not excluded due to the shortage of CYPs and other enzyme activities in the cells. In addition, when exposing the cells to these metabolites, they are unlikely to enter the cells in significant concentrations. On the other hand, it is most likely that troglitazone causes hepatic cell death via apoptosis. Caspase-3 was activated by troglitazone treatment, and pharmacological inhibition of caspase blocked troglitazone-induced cell death (Jung et al. 2007). Apoptosis is a normal physiologic form of cell death and plays a prominent role in liver pathogeneses such as autoimmune liver diseases, viral hepatitis, and drug-induced hepatitis. From this point of view, the cellular, molecular, and *in vivo* responses to troglitazone toxicity will be reviewed in the following sections.

7.1 Mitochondria-Mediated Toxicity

Mitochondria are known to be a source of reactive oxygen species (ROS), suggesting that a direct effect of troglitazone on mitochondrial physiology may play a role in hepatotoxicity (Narayanan et al. 2003). The development of troglitazone-induced toxicity in liver cells could be caused by a reduction of the mitochondrial membrane potential with a concomitant depletion of cellular ATP concentration (Bova et al. 2005; Tirmenstein et al. 2002). Subsequently, it increases the mitochondrial membrane permeability transition and calcium ion (Ca^{2+}) efflux (Masubuchi et al. 2006). The result of these effects on mitochondria is the release of cytochrome c into the cytoplasm and activation of caspases leading to apoptosis (Bova et al. 2005). Using immortalized human hepatocytes, Lim et al. (2008) found that troglitazone rapidly dissipated the mitochondrial inner transmembrane potential, followed by a shift of the redox ratio of mitochondrial thioredoxin-2 (Trx2) toward the oxidized state, and subsequent activation of apoptosis signal-regulating kinase 1 (Ask1). Ong et al. (2007) established heterozygous superoxide dismutase 2 hetero-knockout [Sod2(+/−)] mice as an experimental animal model of silent mitochondrial stress. They found that troglitazone caused liver injury in the high-dose (30 mg/kg/day, i.p.) group, manifested by an approximately twofold increase in serum ALT in Sod2(+/−) but not in wild-type mice. This mouse model could be useful to analyze the dynamics of mitochondrial changes *in vivo* and to investigate the involvement of reactive metabolites in mitochondrial toxicity. Thus, mitochondrial abnormalities could be one of the useful biomarkers of troglitazone-induced idiosyncratic hepatotoxicity.

7.2 Kinase-Mediated Cell Toxicity Pathway

The three well-characterized mammalian mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (Erk), c-Jun N-terminal kinase (JNK), and p38 are regulated by phosphorylation and play important roles in a variety of cellular processes including growth, differentiation, and apoptosis (Johnson and Lapadat 2002). Erk is generally activated by mitogens, while JNK and p38 are preferentially activated by stress and inflammatory cytokines. The most obvious effect of troglitazone on apoptosis is likely via the promotion of JNK, which in turn activates c-Jun by phosphorylation as well as by activation of p38 (Bae and Song 2003). Gardner et al. (2005) and Jung et al. (2007) reported that calcium/calmodulin-dependent kinase II (CaMKII) is a critical regulator of double-stranded RNA-activated protein kinase (PKR)-dependent p38 and eukaryotic initiation factor 2α (eIF2α) phosphorylation in response to endoplasmic reticulum (ER) calcium depletion by troglitazone. Activation of these kinase-signaling pathways is PPAR γ-independent. In addition, troglitazone also causes the induction of Bax, Bad, the cleavage of Bid, and the release of cytochrome c. Moreover, the mitogen-activated protein kinase (MEK) 1/2-ERK1/2 signaling pathway may be implicated in the

growth inhibitory effect by troglitazone in human cancer cell lines (Motomura et al. 2005; Jung et al. 2007). JNK is characterized as a stress-activated protein kinase based on its activation in response to the inhibition of protein synthesis. These reports suggest that troglitazone induces apoptosis via a caspase-dependent mechanism associated with the downregulation of MEK/ERK and upregulation of p38.

Cyclin-dependent kinases (CDKs) are serine-threonine protein kinases that regulate cell cycle progression. These kinases are activated by various cyclins, inhibited by natural inhibitors such as p21, p27, and p18, and are tightly controlled by transcriptional and posttranscriptional modifications (Sherr and Roberts 1999). Bae et al. (2003) reported that troglitazone-induced cell cycle arrest by this pathway, and apoptosis of hepatoma cell lines ware caused G1 cell cycle arrest through the induction of p53 related proteins and the reduction of cyclin D1, phospho-RB and CDK activities.

7.3 Protein Translation-Associated Toxicity

The endoplasmic reticulum (ER) is a major site of protein synthesis, and its inside or lumen is a major site of protein folding (Gething and Sambrook 1992). In mammalian cells, naturally the rate of protein synthesis is rapidly reduced following the induction of apoptosis. The phosphorylation of eIF2α is important in the regulation of selective translation during ER stress and the unfolded protein response (Holcik and Sonenberg 2005). Troglitazone was shown to promote Ca^{2+} release from the ER leading to PERK and PKR activation, phosphorylation of eIF2α, translation inhibition, and growth arrest (Fan et al. 2004; Gardner et al. 2005).

It is known that the ER is a major cellular storage site of Ca^{2+} in the cell, and that ER chaperones play important roles in Ca^{2+} accumulation and release. Any disturbance in the ER homeostasis causes the release of Ca^{2+}, which in turn blocks ER protein processing. This results in the accumulation of incompletely folded proteins and activates the transcription of ER chaperone genes (Liu et al. 1998; Lodish and Kong 1990). We found that troglitazone treatment of hepatoma cell lines led to overexpression of immunoglobulin heavy chain binding protein (BiP), an abundant chaperone protein in the ER (Maniratanachote et al. 2005a). The important role of this chaperone protein was indicated by the phenotypic change in cell viability when BiP expression was inhibited by small interference RNA (Maniratanachote et al. 2005a). This condition rendered cells more susceptible to the toxic effects of troglitazone. Collectively, it might be postulated that troglitazone acts as a chemical stress signal that causes the release of Ca^{2+} from the ER, and that BiP expression is one of the cellular defense mechanisms of the ER in response to troglitazone-induced toxicity.

Ribosomal protein P0 (P0) was found to be one of the targets of troglitazone cytotoxicity in HepG2 cells (Maniratanachote et al. 2006). P0 is known as a phosphoprotein that functions in the protein translation process (Gonzalo et al. 2001). It was found that, rather than its overexpression, dephosphorylation of P0, which could not be prevented by caspase inhibition, occurred in troglitazone-induced cytotoxicity