Increased susceptibility to troglitazone-induced mitochondrial permeability transition in type 2 diabetes mellitus model rat

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ABSTRACT — Troglitazone, a member of the thiazolidinedione class of antidiabetic drugs, was withdrawn from the market because it causes severe liver injury. One of the mechanisms for this adverse effect is thought to be mitochondrial toxicity. To investigate the characteristics of troglitazone-induced liver toxicity in more depth, the toxicological effects of troglitazone on hepatocytes and liver mitochondria were investigated using a rat model of type 2 diabetes mellitus (T2DM). Troglitazone was found to increase mitochondrial permeability transition (MPT) in the liver mitochondria of diabetic rats to a greater extent than in control rats, whereas mitochondrial membrane potential and oxidative phosphorylation were not affected. To identify the factors associated with this increase in susceptibility to MPT in diabetic rats, we assessed the oxidative status of the liver mitochondria and found a decrease in mitochondrial glutathione content and an increase in phospholipid peroxidation. Moreover, incorporation of oxidized cardiolipin, a mitochondrion-specific phospholipid, was involved in the troglitazone-induced alteration in susceptibility to MPT. In conclusion, liver mitochondria display disease-associated mitochondrial lipid peroxidation in T2DM, which facilitates the higher susceptibility to troglitazone-induced MPT. Thus, greater susceptibility of liver mitochondria may be a host factor leading to troglitazone-induced hepatotoxicity in T2DM.

Key words: Diabetes, Troglitazone, Liver mitochondria, Mitochondrial permeability transition, Lipid peroxidation, Cardiolipin

INTRODUCTION

Despite substantial advances in pharmaceutical therapies, many adverse drug reactions (ADRs) still occur, and therefore improvements in therapeutic regimens and the development of safer drugs are both required. Drug-induced liver injury (DILI) is recognized as a major ADR that has significant impact on clinical staff, patients, and pharmaceutical researchers, because in the worst-case scenario it can lead to fulminant liver failure. The majority of ADRs affecting the liver are idiosyncratic, and the relationship between the incidence of DILI and their severity has been thought to depend on three factors: the drug itself (its chemical structure, molecular weight, lipophilicity, and duration of action), environmental factors (alcohol, diet, coffee, tobacco, the microbiome, and toxins), and host factors (age, gender, genetics, immune status, metabolism, and concurrent disease). Among the host factors that are considered to modify the risk of hepatotoxicity are disease conditions, genetic variants, and nutrition (Eichelbaum et al., 2006; Du et al., 2013). However, it is insufficient to study drug-host interactions alone, because little research regarding host factors has been conducted.

Thiazolidinediones, including troglitazone and pioglitazone, are antidiabetic drugs that target peroxisome proliferator-activated receptor (PPAR) γ. It has been suggested that their on-target effects are not involved in their cellular toxicity (Yamamoto et al., 2001; Bae et al., 2003), but troglitazone can induce severe hepatotoxicity and was voluntarily withdrawn from the market in 2000. Although the mechanisms of troglitazone-induced hepatotoxicity in T2DM, which facilitates the higher susceptibility to troglitazone-induced MPT. Thus, greater susceptibility of liver mitochondria may be a host factor leading to troglitazone-induced hepatotoxicity in T2DM.
toxicity have been characterized as formation of reactive metabolites, oxidative stress, mitochondrial toxicity, bile acid accumulation and mitochondrial permeability transition (MPT) (Kassahun et al., 2001; Bova et al., 2005; Narayanan et al., 2003; Ogimura et al., 2017; Masubuchi et al., 2006), little research has been conducted on the host characteristics in type 2 diabetes mellitus (T2DM), involved in troglitazone-induced hepatotoxic effects.

A previous prospective study of patients with DILI showed that there was higher mortality in DILI patients with pre-existing liver disease than in those without liver disease, and that those with known pre-existing liver disease had a higher prevalence of T2DM (Chalamani et al., 2015). As T2DM is characterized by altered energy metabolism and mitochondrial function, mitochondrial dysfunction can result in severe liver injury. Indeed, ~80% of the drugs presently prescribed with Food and Drug Administration black box warnings can induce mitochondrial toxicity (Dykens and Will, 2007). Moreover, mitochondria are a major source of reactive oxygen species (ROS) generated by dysfunction of respiratory chain complexes due to disease and drug toxicity (Pessayre, 2007; Hu et al., 2015). In investigation of using animal model of reduced anti-oxidative capacity, hepatotoxic drug induced more severe liver injury than in normal animal (Ong et al., 2007). Taken together, liver mitochondrial function may play crucial roles in developing DILI.

From these observations, we hypothesized that the susceptibility of liver mitochondria to drug-induced liver injury might be higher in T2DM, leading to a greater prevalence of DILI.

To test this hypothesis, in this study, we evaluated liver mitochondrial function between control rat and T2DM model rat to compare susceptibility to troglitazone, and then measured mitochondrial redox status to elucidate factors of alteration in susceptibility. Moreover, cardiolipin (CL), which is a mitochondrial specific phospholipid that contains four fatty acid chains, can be easily oxidized by mitochondrial ROS, because most of fatty acid chains are unsaturated (e.g. linoleic acid). It has been suggested that alteration of CL quantity and quality could change mitochondrial membrane fluidity, and the complexes activity. Therefore, we focused on oxidation of mitochondrial lipid in T2DM model which may cause differences of susceptibility to troglitazone.

MATERIALS AND METHODS

Materials
Troglitazone, pioglitazone, and rhodamine-123 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Phenformin was from Sigma-Aldrich Corp. (St. Louis, MO, USA), and calcein-AM was from Nacalai Tesque, Inc. (Kyoto, Japan). Hoechst 33342 was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA), and bovine heart CL was from Avanti Polar Lipids (Alabaster, AL, USA). Other general reagents were of analytical grade and obtained from conventional commercial sources.

Animals
Five-week-old male Zucker Diabetic Fatty (ZDF) lean (+/+) and ZDF fa/fa rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). ZDF fa/fa rats develop T2DM, while ZDF lean (+/+ ) rats represent ZDF +/+ or fa/+ rats and normal blood glucose level, and then these are used as control rats not to distinguish between ZDF +/+ and ZDF fa/+ rats. Wistar rats, for the preparation of phospholipid-fused mitochondria, were purchased from SLIC Japan, Inc. (Tokyo, Japan). Animals were maintained on standard laboratory chow (Oriental Yeast Co., Tokyo, Japan) and water ad libitum, under a reverse dark-light cycle. Animals were fasted for > 18 hr before all experiments. All animals were treated humanely in accordance with guidelines issued by the National Institutes of Health (Bethesda, MD, USA), and all procedures were approved by the Animal Care Committee of Chiba University.

Hepatocyte isolation, purification, and culture
Rat hepatocytes were isolated using a two-step perfusion method, as reported previously (Takemura et al., 2016). The cells were seeded onto collagen-coated tissue culture plates at a density of 4.125 × 10⁴ cells/well in 0.1 mL of plating medium in 96-well plates. Cells were incubated in a humidified atmosphere containing 5% CO₂ at 37°C and allowed to attach for 2 hr, after which the medium was aspirated to remove unattached cells and replaced with fresh plating medium.

Isolation of liver mitochondria
Liver mitochondrial fractions were prepared according to a previously described method (Schneider and Hogeboom, 1950), with modifications. Livers were isolated and placed in ice-cold isolation medium (250 mM sucrose, 10 mM HEPES-KOH (pH 7.2)) containing 0.5 mM EGTA, after which they were cut into small cubes with scissors, placed into 30 mL of medium, and homogenized 5 times in a Potter homogenizer. Homogenates were centrifuged at 770 × g for 5 min at 4°C. The resulting supernatant was decanted and further centrifuged at 9,800 × g for 10 min at 4°C. The supernatant was dis-
cared, and the pellets were suspended in 10 mL of ice-cold isolation medium containing 0.3 mM EGTA. This suspension was centrifuged at 4,500 × g for 10 min at 4°C, the supernatant was discarded, and the pellets were resuspended in 5 mL of ice-cold isolation medium containing 0.3 mM EGTA. Then, the suspension was centrifuged using a centrifugal force ranging from 2,000 × g for 2 min to 4,500 × g for 8 min and maintained at 4°C. The final mitochondrial pellet was suspended in 0.7 mL of the ice-cold isolation medium. The protein concentration of the mitochondrial fraction was determined by the method of Lowry et al. (1951) using a Multiskan JX system (MTX Lab Systems, Vienna, VA, USA). Intact mitochondria (respiratory control ratio (RCR) > 4 with glutamate/malate as substrate) were used for all experiments.

**Measurement of MPT pore opening in rat primary hepatocytes**

Twenty-four hours after plating, test compounds were dissolved in Williams’ medium E, and cells were exposed for 6 hr at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Measurement of MPT pore opening activity in rat primary hepatocytes was performed using calcine-AM, CoCl₂, and Hoechst 33342, as reported previously (Sekine et al., 2013). After the treatment with test compounds, cells were loaded with 1 μM calcine-AM, 1 mM CoCl₂, and 1 μM Hoechst 33342 to correct for cell number. The loss of mitochondrial calcine and Hoechst 33342 nuclear fluorescence was measured using a fluorescence microscope (BZ-X700, Keyence, Osaka, Japan) under basal conditions.

**Measurement of MPT in isolated mitochondria**

Mitochondria (0.5 mg protein/mL) were preincubated in reaction medium (125 mM sucrose, 65 mM KCl, 5 mM succinate, and 10 mM HEPES-KOH (pH 7.4)) at 30°C in the presence of 10, 20, or 50 μM CaCl₂. The reaction was initiated by the addition of various concentrations of test compound and continued for 5 min. The reaction medium was then immediately centrifuged (16,000 × g, 30 sec, 25°C), and the fluorescence intensity of the supernatant was measured using an excitation wavelength of 485 nm and an emission wavelength of 535 nm. MMP was calculated using the Nernst equation, as described previously (Shitara et al., 2013). The following equation was used: $\Delta\Psi = 59 \times \log[Rhod123]_{in}/[Rhod123]_{out}.$

**Measurement of oxygen consumption rate (OCR) in isolated mitochondria**

Mitochondrial respiratory function was represented by OCR and measured using a fluorescent oxygen probe (Presens, Regensburg, Germany) according to a previously described method (Shitara et al., 2013), with modifications. Mitochondria (1 mg protein/mL) were preincubated at 30°C in 0.5 mL of respiration medium (225 mM sucrose, 10 mM KCl, 5 mM MgCl₂, 5 mM potassium phosphate, 0.5 mM EDTA, and 20 mM Tris-HCl) containing 5 mM glutamate plus 1 mM malate, or 4 mM succinate, as respiratory substrates. Then, 0.2 mM ADP, followed by test compounds, were added to samples prepared as described above. State 3 and state 4 respiration rates were measured in the presence (state 3) and after exhaustion (state 4) of ADP, respectively. RCR means a capacity for oxidative phosphorylation to synthesize ATP and the value was calculated as the ratio of the state 3/state 4 respiration rates.

**Measurement of reduced/oxidized glutathione (GSH/GSSG) in isolated mitochondria and liver homogenates**

Samples were mixed with cold EDTA-metaphosphate solution and centrifuged at 15,000 × g for 2 min at 4°C. The supernatant was incubated with reaction medium (1 mM 5,5’-dithiobis-2-nitrobenzoic acid, 0.34 mM NADPH, 104.4 mM NaH₂PO₄, and 4.6 mM EDTA (pH 7.4)) containing GSSG reductase at room temperature. The reduced/oxidized glutathione (GSH/GSSG) concentration of the samples was determined from a standard curve using the enzymatic method and a Multiskan JX system (Baker et al., 1990).

**Measurement of thiobarbituric acid-reactive substances (TBARS) in isolated mitochondria and liver homogenates**

Samples were washed with cold PBS, and mixed with
3.86% perchloric acid and 20 mM thiobarbituric acid reagent. The mixture was heated for 15 min at 100°C and centrifuged at 1,600 × g for 15 min at room temperature. The supernatant was collected, and its absorbance was measured at 480-570 nm wavelength using a Shimadzu UV-2550 spectrophotometer.

**Extraction of lipids from isolated mitochondria and high-performance liquid chromatography (HPLC) analysis**

Total lipid was extracted from rat liver mitochondria using the Bligh-Dyer method (Bligh and Dyer, 1959). Chloroform/methanol (2:1, v/v) containing 0.05% butylated hydroxytoluene and 10 mM HCl was added to the samples, which were then vortexed. The mixture was centrifuged at 1,600 × g for 5 min at room temperature, and the lower phase was collected and dried under N₂ gas. The sample was re-dissolved in methanol and analyzed by HPLC using a normal phase column (3 μm, 4.6 × 250 mm) at 206/235 nm to monitor phospholipid and its hydroperoxide based on conjugated dienes (Imai et al., 2003). The samples were eluted with a flow rate of 0.5 mL/min at 40°C using hexane/2-propanol/methanol/water (5:15:2:3, v/v/v/v) as a mobile phase.

**Measurement of MPT using vesicle-fused mitochondria**

The fusion of vesicles with isolated liver mitochondria was conducted according to a previously described method (Imai et al., 2003). The prepared mitochondrial fusions were resuspended in MPT assay medium (125 mM sucrose, 65 mM KCl, 5 mM succinate and 10 mM HEPES-KOH (pH 7.4)). Cardiolipin hydroperoxide (CLOOH) was prepared by auto-oxidizing thin films of bovine heart CL at 37°C overnight.

**Statistical analysis**

Data are presented as mean ± S.E. Statistical comparisons were conducted using Student’s t-test (for two groups) or ANOVA followed by Dunnett’s test (for multiple groups).

**RESULTS**

**Animal characteristics**

In ZDF fa/fa rats, body mass increased more than in ZDF lean rats during the feeding period, and by 8 weeks of age ZDF fa/fa rats were significantly heavier (Table 1). Similarly, ZDF fa/fa rats had higher blood glucose at 12 weeks of age (Table 1). Despite being fasted for >18 hr before liver isolation, blood glucose was significantly higher in ZDF fa/fa rats than controls (Table 1; ZDF lean rats, 133 ± 7 mg/dL; ZDF fa/fa rats, 354 ± 39 mg/dL). Higher liver mass (Table 1; ZDF lean rats, 2.6 ± 0.1% body weight; ZDF fa/ra rats, 4.5 ± 0.2% body weight) and the presence of many macroscopic white spots on the liver, likely indicative of lipid deposition (Fig. 1), were observed in the ZDF fa/ra rats. These findings, indicative of hyperglycemia and steatosis, were consistent with previously published data (Etgen and Oldham, 2000) and confirmed that ZDF fa/ra rats represent a suitable model of T2DM for this study.

**Analysis of MPT pore opening activity in primary cultures of rat hepatocytes**

In previous reports, troglitazone induced MPT pore opening in freshly isolated mouse liver mitochondria (Masubuchi et al., 2006) and caused structural abnormalities in mitochondrial matrix and in the inner/outer membrane of HepG2 cells (Tirmenstein et al., 2002). MPT pores regulate the passage of cytochrome C, apoptosis-inducing factor, and Ca²⁺ through the mitochondri-

**Table 1. Body weights, liver weights and blood glucose levels of ZDF rats.**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Body weight (g)</th>
<th>Blood glucose (mg/dL)</th>
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<tbody>
<tr>
<td>8 weeks Body weight</td>
<td>228 ± 3</td>
<td>118 ± 7</td>
</tr>
<tr>
<td>12 weeks Body weight</td>
<td>324 ± 6</td>
<td>116 ± 9</td>
</tr>
<tr>
<td>Assay (14-15 weeks)</td>
<td>353 ± 7</td>
<td>342 ± 10</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.E. (n = 4).

ZDF rats at 8 and 12 weeks of age were measured under non-fasting condition.

After ZDF rats aged 14-15 weeks were fasted overnight, their liver and blood were collected.

*; significantly different from ZDF lean group in each row (p < 0.05), **; (p < 0.01).
al membrane. To elucidate whether greater susceptibility to troglitazone-induced MPT was associated with T2DM, we conducted Co²⁺-calcein assays in hepatocytes isolated from the livers of ZDF lean and ZDF fa/fa rats. Co-treatment with Co²⁺, which cannot be incorporated into mitochondria, successfully quenched the fluorescence generated using cytosolic calcein, as previously demonstrated in HepG2 cells (Tan et al., 2009). As shown in Fig. 2, mitochondrial calcein fluorescence was not altered by treatment with troglitazone (~10 μM for 6 hr) in hepatocytes isolated from ZDF lean rats, whereas it was significantly lower after troglitazone treatment of ZDF fa/fa rat hepatocytes. These results demonstrate that liver mitochondria from ZDF fa/fa rats are more susceptible to troglitazone-

![Fig. 1. Liver samples of ZDF rats in assay condition. After ZDF rats were fasted overnight, their livers were collected.](image)

![Fig. 2. Concentration-dependent effects of troglitazone on MPT pore opening in ZDF rat hepatocytes. (A) Representative images of calcein (green) and hoechst33342 (blue) fluorescence. (B) Quantitative results of calcein fluorescence intensity normalized to individual cells. Isolated primary hepatocytes were loaded with calcein-AM, cobalt chloride and hoechst33342 for 30 min, following incubating with troglitazone for 6 hr. MPT pore opening was determined by measuring intensity of calcein fluorescence in the mitochondria quenched by cobalt and normalizing cell number of hoechst33342. Each column represents the mean ± S.E. (n = 6 from two experiments). #; significantly different from ZDF lean rats (p < 0.05) Tro; Troglitazone.](image)
induced MPT.

**Swelling of isolated liver mitochondria**

MPT is thought to be one of the mechanisms leading to cell death. We next investigated whether the susceptibility of MPT to induction by antidiabetic drugs was also detectable in isolated liver mitochondria. Troglitazone potently induced swelling of liver mitochondria isolated from ZDF fa/fa rats in a concentration-dependent manner, but this was not observed in liver mitochondria from ZDF lean rats (Fig. 3A). We next evaluated the effects of other antidiabetic drugs (pioglitazone and phenformin). Pioglitazone is also a thiazolidinedione, but has previously been shown not to induce MPT (Masubuchi et al., 2006). Phenformin is a member of the biguanide class of antidiabetic drugs and inhibits complex I activity of the mitochondrial electron transport chain, but does not potentiate MPT pore opening (Drahota et al., 2014). Like troglitazone, susceptibility to mitochondrial swelling after pioglitazone or phenformin treatment was slightly but significantly potentiated in ZDF fa/fa rats but not in ZDF lean rats (Figs. 3B and 3C). Thus, greater susceptibility to antidiabetic drugs-induced MPT pore opening was observed in both liver mitochondria and hepatocytes isolated from ZDF fa/fa rats.

**MMP and RCR in isolated liver mitochondria**

Changes in MMP (Δψ) mainly represent changes in the proton gradient across the inner mitochondrial membrane, which can be induced by respiratory chain inhibitors. In addition, the collapse of MMP also leads to mitochondrial swelling and rupture of the outer membrane. Because susceptibility to MPT induction was greater in ZDF fa/ fa rats, the susceptibility to changes in MMP might also be enhanced by troglitazone. To investigate this possibility, we compared the change in MMP induced by antidiabetic drugs in isolated liver mitochondria from ZDF fa/fa and lean rats, and found that the MMP-dependent incorporation of rhodamine-123 was not different between these strains (Fig. 4). Troglitazone potentiated a decrease in MMP in ZDF lean rats in a concentration-dependent manner, but the susceptibility of MMP to a troglitazone-induced decrease was comparable in ZDF fa/fa and lean rats (Fig. 4). As for troglitazone, pioglitazone and phenformin decreased MMP similarly in both strains.

RCR indicates the membrane integrity of isolated mitochondria and their capability to perform efficient oxidative phosphorylation (OXPHOS). Mitochondria produce ATP as the end-product of electron flux through respiratory chain complexes, ultimately driven by the proton gradient across the mitochondrial inner membrane. However, troglitazone is an inhibitor of mitochondrial OXPHOS complexes, and therefore it disrupts mitochondrial respiration (Nadanaciva et al., 2007). To investigate the effects of antidiabetic drugs on the respiratory function of liver mitochondria isolated from ZDF rats, RCR values were determined.

Control RCRs of liver mitochondria with glutamate/malate and succinate as substrates for complex I and complex II were not significantly different between ZDF rat strains (Figs. 5A and 5B). There were also no significant differences in mitochondrial oxygen consumption capacity in states 3 and 4 between ZDF lean and fa/fa rats (Supplemental Fig. 1). Troglitazone treatment had an inhibitory effect and caused uncoupling, whereas pioglitazone had a lesser effect. However, phenformin has the poten-

![Fig. 3.](image-url) Concentration-dependent effects of diabetes drugs on mitochondrial swelling in ZDF rat liver mitochondria. Mitochondrial swelling was determined by incubating liver mitochondria with 10 μM Ca²⁺ plus diabetes drugs (A-C). Each plot represents the mean ± S.E. (n = 4). *; significantly different from 0 μM diabetic drug (p < 0.05), **; (p < 0.01), #; significantly different from ZDF lean rats (p < 0.05).
Increased susceptibility to troglitazone-induced MPT in T2DM rats

Fig. 4. Concentration-dependent effects of diabetes drugs on mitochondrial membrane potential in ZDF rat liver mitochondria. $\Delta \psi$ was determined by incubating liver mitochondria with diabetes drugs and succinate in the presence of rhodamine123. Each column represents the mean + S.E. (n = 4). *; significantly different from control group (p < 0.05), **; (p < 0.01).

Fig. 5. Concentration-dependent effects of diabetes drugs on mitochondrial oxygen consumption in ZDF rat liver mitochondria. RCR was determined by incubating liver mitochondria with diabetes drugs and glutamate/malate (A) or succinate (B). Each column represents the mean + S.E. (n = 4). *; significantly different from control (p < 0.05), **; (p < 0.01).

tial to inhibit complex I of the mitochondrialOXPHOS pathway. In liver mitochondria from ZDF lean rats treated with phenformin, OCR in state 3 tended to be lower, while that in state 4 was significantly enhanced in a concentration-dependent manner, resulting in a decrease in RCR (Fig. 5 and Supplemental Fig. 1). However, in ZDF fa/fa rats, these drugs had equivalent inhibitory effects on the mitochondrial respiratory chain in ZDF strains (Fig. 5 and Supplemental Fig. 1). Overall, these results indicate that the susceptibility to antidiabetic drug-induced MPT, but not MMP depletion, and to lower mitochondrial respiration activity, was significantly greater in liver mitochondria isolated from ZDF fa/fa rats.

Glutathione status and lipid peroxidation in ZDF rats

To investigate the mechanism of the increased susceptibility to troglitazone-induced MPT pore opening, we
focused on oxidative stress, because mitochondria are the principal source of ROS. To determine mitochondrial oxidative stress status, GSH and its oxidized form (GSSG) were assayed in liver mitochondria from ZDF rats. Lower GSH content was observed in the mitochondrial fraction of ZDF fa/fa rats versus lean rats (Fig. 6A). GSSG/GSH ratio had tendency to increase in ZDF fa/fa rat mitochondria (Fig. 6C). However, there were no differences between strains in oxidative stress markers in the liver homogenate fraction (Fig. 6).

We next measured lipid peroxide levels in mitochondria and found that TBARS formation was higher in mitochondria but not in the total homogenate fraction prepared from ZDF fa/fa rat liver (Fig. 7A). To further evaluate the mitochondrial peroxide status of ZDF fa/fa rats, oxidation of mitochondrial phospholipids was measured by HPLC analysis. Consistent with the differences in TBARS formation, HPLC analysis of mitochondrial phospholipids in isolated mitochondria showed that per-oxidative phospholipid content was higher in ZDF fa/fa than in lean rats (Fig. 7B). These data indicate that mild disturbance of mitochondrial redox status with diabetes

![Fig. 6](image1)

**Fig. 6.** GSH and GSSG contents in ZDF rat liver mitochondria and homogenate. Each column represents the mean ± S.E. (n = 4). Mt; mitochondrial fraction, Hm; homogenate fraction. #; significantly different from ZDF lean rats (p < 0.05).

![Fig. 7](image2)

**Fig. 7.** Lipid and phospholipid peroxidation in ZDF rat liver. (A) TBARS formation in liver mitochondria and homogenate. (B) Relative peroxidative phospholipid content in liver mitochondria. Peak areas at 206 nm and 235 nm analyze non-peroxidative and peroxidative phospholipid, respectively. Peak area ratio (235/206 nm) shows relative peroxidative phospholipid content. Each column represents the mean ± S.E. (n = 4). Mt; mitochondrial fraction, Hm; homogenate fraction. #; significantly different from ZDF lean rats (p < 0.05).
progression alters susceptibility to drug-induced effects, especially the susceptibility to antidiabetic drug-induced MPT induction.

MPT assay using fused mitochondria containing CL

We hypothesized that the greater susceptibility to antidiabetic drug-induced MPT pore opening in diabetic rats was attributable to oxidative stress. The possible causes of MPT induction include mitochondrial calcium overload, disruption of OXPHOS, rupture of the mitochondrial outer membrane, and release of proapoptotic factors. CL, which is mainly located in the inner membranes of mitochondria, is a phospholipid that is strongly bound to OXPHOS complexes, MPT pore-associated protein, and cytochrome c and regulates biosynthetic pathways. A previous report showed that CL peroxidation is associated with MPT pore opening and release of cytochrome c that led to apoptotic cell death (Imai et al., 2003). Therefore, we next investigated the role of CL in the effects of troglitazone on MPT susceptibility.

Isolated rat liver mitochondria from normal Wistar rats were preincubated with micellized phospholipid containing CL or CLOOH, so that they would incorporate these exogenous CL species, and then a swelling assay was performed. This showed that the susceptibility to Ca²⁺-induced mitochondrial swelling was lower in mitochondria containing CL than in control mitochondria. By contrast, incorporation of CLOOH increased the susceptibility to Ca²⁺-induced mitochondrial swelling (Fig. 8A). In addition, CLOOH-containing mitochondria responded to troglitazone treatment with a large amount of mitochondrial swelling (Fig. 8B). These results indicate that the oxidized form of CL enhances susceptibility to troglitazone-induced MPT.

DISCUSSION

It is recognized that not only the drug itself, but also host factors (for example, the presence of disease, genetic variants, and nutrition) represent important risk factors for DILI. However, these host factors have not been comprehensively investigated. Following a previous report that indicated that T2DM could be a risk factor for chronic liver disease, we hypothesized that T2DM would be a host risk factor for DILI. In support of this contention, patients with T2DM also commonly have liver disease (Chalasani et al., 2015).

ZDF fa/fa rats have been widely used as an animal model of obesity with T2DM. This strain has a mutation in the leptin receptor gene and a defect in pancreatic β cells, which induce obesity, hyperglycemia, hyperlipidemia, and insulin resistance. In the current study, we demonstrated that the susceptibility to antidiabetic drug-induced MPT pore opening is greater in ZDF fa/fa rats than in ZDF lean rats. But a previous study using other models, namely, streptozotocin-induced diabetic rats and non-obese Goto-Kakizaki rats (models of type 1 and type...
2 diabetes, respectively), demonstrated that MPT susceptibility was reduced by metabolic adaptation (Ferreira et al., 2003). The discrepancy in findings between ZDF rats and these other models is likely to be explained by the presence or absence of obesity, and Fig. 7 shows that liver mitochondria isolated from ZDF fa/fa rats had comparatively high levels of lipid/phospholipid peroxidation.

Because mitochondria have a central role in ATP generation, loss of function is considered to be a key mechanism in the development of drug-induced toxicity. Therefore, we assessed mitochondrial swelling, MMP, and OCR in diabetic rat liver to determine whether T2DM might influence mitochondrial function and also susceptibility to drug-induced mitochondrial toxicity. Troglitazone induced a decrease in mitochondrial Ca\(^{2+}\) retention, which is an indicator of MPT pore opening, in hepatocytes of ZDF fa/fa rats (Fig. 2). Consistent with this event in hepatocytes, mitochondrial swelling was strongly induced by troglitazone in the liver mitochondria of ZDF fa/fa rats (Fig. 3A). These data indicate that troglitazone-induced MPT was exacerbated in diabetic rat liver. In contrast to troglitazone, pioglitazone, use of which carries a lower risk of DILI in the clinical setting, caused less mitochondrial parameters to troglitazone were comparable between lean and obese ZDF rats. While one report demonstrated that a drug that induces MPT, such as diclofenac, would also reduce MMP (Masubuchi et al., 2002), another report showed that a representative reagent (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone) reduced MMP but was less potent at inducing MPT (Petronilli et al., 2001). These discrepant results suggest that there are independent mechanisms regulating MPT and MMP.

It is reported that MPT pore opening activity is regulated by various mechanisms (for example, calcium signaling and TNF-α signaling), which may ultimately lead to cell death (Brookes et al., 2007; Hossain et al., 2009). We hypothesized that the differences in susceptibility to MPT induction were caused by mitochondrial oxidative stress. Previously, it was demonstrated that troglitazone strongly induces ROS-mediated MPT potency in mitochondria (Okuda et al., 2010), because ROS generation accelerated MPT pore opening, but this was prevented by the addition of an antioxidant (Zorov et al., 2000). These findings suggest that troglitazone would have promoted ROS-mediated MPT induction in the liver of diabetic rats. In this study, we showed that increased susceptibility to troglitazone-induced MPT is caused by increase of ROS level associated with T2DM and decrease of GSH level in liver mitochondria of ZDF fa/fa rats. In case of repetitive administration, troglitazone may induce long-term ROS generation and reduction of GSH in liver mitochondria, and the specific ROS generation may be also a factor of MPT induction. Diabetes is one of the most common metabolic diseases in humans, and hyperglycemia exacerbates oxidative stress and causes a decline in antioxidant capacity, leading to the development or exacerbation of this disease and its complications. The development of diabetes is associated with mitochondrial oxidative damage and a reduction in mitochondria biogenesis secondary to ROS generation through the OXPHOS pathway, leading to mitochondrial dysfunction and insulin resistance. Although no significant difference in mitochondrial function between isolated liver mitochondria from ZDF rat strains was observed, OCR and MMP were slightly lower in ZDF fa/fa than in ZDF lean rats (Figs. 4 and 5). Consistent with these observations, ZDF fa/ fa rats demonstrated significantly lower GSH content in liver mitochondria but not in the liver homogenate fraction. Furthermore, a higher level of lipid/phospholipid peroxidation was also observed in liver mitochondria isolated from ZDF fa/fa rats (Fig. 7). These results indicate that the chronic inhibition of mitochondrial OCR in diabetes may induce mitochondrion-specific oxidative stress. From a perspective in potencies which troglitazone itself induces oxidative stress and decreases GSH content (Narayanan et al., 2003), troglitazone may further decrease GSH level in liver mitochondria of ZDF fa/fa rats. This possibility needs to be examined in the future study.

Because there was a difference in the susceptibility to MPT induction between ZDF rat strains, we focused on the role of lipids, especially the mitochondrion-specific species CL. Most mitochondrial membrane phospholipids are composed of phosphatidylcholine, phosphatidylethanolamine, and CL, and their composition varies between tissues, organs, and animal species (Ardail et al., 1990). CL has important roles, such as the regulation of MPT pore-associated proteins to induce apoptotic signaling, of OXPHOS activities to maintain cellular ATP content, and of mitochondrial remodeling to regulate mitochondrial quality (Claypool and Koehler, 2012). In this study, mitochondrial membrane composition was modified to include phospholipid containing either CL or oxidized CL (CLOOH). The introduction of CLOOH into mitochondria significantly increased troglitazone-in-
Reduced MPT pore opening (Fig. 8B). Because CL is mostly composed of unsaturated fatty acids and mainly distributes to the inner mitochondrial membrane (Paradies et al., 2009), mitochondrial-generated ROS could attack CL molecules. It was previously reported that total glutathione levels were lower and there was an increase in protein adducted with 4-hydroxynonenal, the end product of oxidized CL, in the liver of T2DM patients (Valle et al., 2012). These results imply that the greater susceptibility to troglitazone-induced MPT pore opening in T2DM rats might be attributable to alterations in CL caused by oxidative stress during diabetes. Interestingly, it was also reported that troglitazone-induced MPT was caused by ROS-derived process and Ca^{2+}-independent phospholipase A_2 (iPLA_2) derived process (Okuda et al., 2010). iPLA_2 mainly hydrolyzes glycerophospholipid and catalyzes to free fatty acid and lysophospholipid, and CL is also a substrate for iPLA_2. In this study, CL-containing mitochondria decreased the susceptibility to troglitazone-induced MPT compared to control mitochondria (Fig. 8B). Although we did not measure iPLA_2 activity of liver mitochondria in T2DM, further investigations should address the effect of the quantity and/or quality of CL in isolated mitochondria on the mechanisms of drug-induced mitochondrial toxicity, especially MPT susceptibility.

Taken together, we could suggest mechanism for increasing susceptibility to troglitazone-induced MPT in ZDF fa/fa rats. In mitochondria from ZDF lean rats, troglitazone by itself causes oxidative stress to induce MPT pore opening through mechanisms of membrane peroxidation and ROS generation. In mitochondria from ZDF fa/fa rats, T2DM is responsible for imbalanced mitochondrial state between excessive generation of ROS and reduced anti-oxidant defense, and exacerbates oxidative stress such as ROS production. Then, these situations have a tendency to induce MPT and result in increasing susceptibility to MPT-induced drugs, especially troglitazone (Fig. 9).

In conclusion, susceptibility of MPT pore opening to troglitazone was greater in hepatocytes and liver mitochondria isolated from a rat model of T2DM, likely as a result of the lower GSH and higher lipid peroxide levels associated with disease progression. The enhanced mitochondrial toxicity, especially MPT induction, in T2DM, could be a predisposing factor for troglitazone-induced hepatotoxicity.

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