Mild depolarization is involved in troglitazone-induced liver mitochondrial membrane permeability transition via mitochondrial iPLA2 activation

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ABSTRACT — Troglitazone, the first peroxisome proliferator-associated receptor γ agonist developed as an antidiabetic drug, was withdrawn from the market due to idiosyncratic severe liver toxicity. One proposed mechanism by which troglitazone causes liver injury is induction of mitochondrial membrane permeability transition (MPT), which occurs in a calcium-independent phospholipase A2 (iPLA2)-dependent manner at a concentration of 10 µM. MPT, induced by opening of the MPT pore, leads to the release of cytochrome c and consequent apoptosis or necrosis. In the present study, we aimed to clarify the mechanism of troglitazone-induced MPT in more detail using isolated rat liver mitochondria. We focused on extra-mitochondrial Ca\(^{2+}\) and membrane potential as triggers of iPLA2 activation or MPT induction. As a link between iPLA2 and MPT, we focused on cardiolipin (CL), a unique, mitochondria-specific phospholipid with four acyl chains that affects respiration, the morphology, and other mitochondrial functions. We found that (1) Ca\(^{2+}\) release from the mitochondrial matrix was induced prior to troglitazone-induced onset of MPT, (2) released Ca\(^{2+}\) was involved in troglitazone-induced MPT, (3) mild depolarization (approximately 10%) may be a trigger of troglitazone-induced MPT and (4) enhanced decomposition of CL following mitochondrial iPLA2 activation might mediate troglitazone-induced MPT.

Key words: Troglitazone, Drug-induced liver injury, Mitochondrial membrane permeability transition, Calcium-independent phospholipase A2, Cardiolipin

INTRODUCTION

Troglitazone, a peroxisome proliferator-associated receptor (PPAR) γ agonist, is an antidiabetic drug that was withdrawn from the market in 2000 due to severe idiosyncratic liver toxicity (Graham et al., 2003). According to clinical reports, massive necrosis was observed in livers of patients, along with a significant increase in alanine transaminase (ALT) (Shibuya et al., 1998; Li et al., 2000). In an animal model, Ong and colleagues reproduced troglitazone-induced liver injury using superoxide dismutase 2 (Sod2) heterozygous knockout mice and observed apoptosis in liver tissues (Ong et al., 2007). Moreover, in vitro, troglitazone suppresses cell proliferation and induces apoptosis in rat hepatocytes (Toyoda et al., 2001) and HepG2, human hepatocellular carcinoma cells (Zhou et al., 2008).

Since other thiazolidinediones with stronger affinity for PPAR γ such as pioglitazone present a lower risk of liver injury, hepatotoxicity caused by troglitazone is considered to be an off-target effect (Shiau et al., 2005). Troglitazone (1) suppresses bile canalicular formation (Takemura et al., 2016), (2) inhibits mitochondrial respiration (Tirmenstein et al., 2002), and (3) induces mitochondrial membrane permeability transition (MPT) (Masubuchi et al., 2006). Moreover, reactive metabolites of troglitazone such as sulfate and/or quinone body are also considered to be involved in hepatotoxicity. Troglitazone sulfate inhibits the bile salt export pump (BSEP), the major transporter of bile acid secretion from hepatocytes (Funk et al., 2001), and troglitazone quinone binds to intracellular macromolecules (Kassahun et al., 2001). These are believed to be the mechanisms underpinning troglitazone-induced liver injury, but the exact molecular
pathways are not fully understood.

Mitochondrial dysfunction is one of the most important factors related to drug-induced liver injury (DILI). Approximately half of all drugs categorized under the “black-box warning” of the US Food and Drug Administration (FDA) are reported to exhibit mitochondrial toxicity (Dykens and Will, 2007). Thus, we particularly focused on mitochondrial impairment as a mechanism of troglitazone-induced liver injury. MPT is caused by opening of the MPT pore, the pore constructed from the c-ring of FoF1 ATP synthase in the inner membrane (Biasutto et al., 2016). The opening of MPT pore is stimulated by cyclophilin D and regulated in ATP synthasome, which includes ATP synthase and adenine nucleotide translocase (ANT) and inorganic phosphate carrier in the inner membrane (Halestrap and Richardson, 2015). We consider MPT to be one of the key events in liver injury because apoptosis is observed in a troglitazone-induced liver injury model (Ong et al., 2007). Elucidation of the mechanism via which troglitazone induces MPT will also be beneficial for developing next-generation drugs with the same mechanism of action.

A previous report showed that troglitazone-induced MPT is mediated by calcium-independent phospholipase A2 (iPLA2) activation in isolated rat liver mitochondria (Okuda et al., 2010). However, it is not yet clear (1) how troglitazone activates iPLA2, and (2) how iPLA2 activation leads to MPT. It is known that troglitazone induces a slight decrease in membrane potential, and decreases the ability of mitochondria to retain accumulated Ca2+ in mouse liver mitochondria (Masubuchi et al., 2006). Mitochondrial iPLA2 is reportedly activated by exogenous Ca2+ addition (Moon et al., 2012). Thus, we deduce that a change in status of Ca2+ is involved in iPLA2 activation.

Since iPLA2 is an enzyme that degrades phospholipids into lyso-phospholipids and fatty acids, we focused on cardiolipin (CL) as a phospholipid of potential relevance to the MPT pore. CL is a unique, mitochondria-specific phospholipid with four acyl chains that accounts for ~20% of phospholipids in the inner mitochondrial membrane (Horvath and Daum, 2013) and its deacylation is promoted by iPLA2 (Hsu et al., 2013; Monteiro et al., 2013). CL contributes to maintaining mitochondrial structure and function, including the integrity of the respiratory super-complexes (Zhang et al., 2002), contact sites (Ardail et al., 1990) and also contributes to oligomerization of ATP synthase (Laage et al., 2015) and binding to and maintaining ANT conformation (Nury et al., 2005). Moreover, oxidation of CL is reported to be involved in Ca2+-induced MPT (Petrosillo et al., 2004). Based on existing knowledge, we hypothesized that decomposition of CL by iPLA2, leads to MPT, and confirmed the hypothesis in this study.

MATERIALS AND METHODS

Reagents

Troglitazone and bromoenol lactone (BEL) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Cyclosporine A (CsA) was purchased from Wako Pure Chemicals (Osaka, Japan). Carboxyl cyanide m-chlorophenyl-hydrazone (CCCP) was purchased from Sigma Aldrich (St. Louis, MO, USA). Arsenazo III was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan).

Animals

All studies were conducted in accordance with the Principles of Laboratory Animal Care as adopted and promulgated by the National Institutes of Health, USA, and the Guidelines for Animal Study provided by Chiba University. All protocols were approved by the Chiba University Institutional Animal Care and Use Committee. Wistar rats (5-7 weeks old) were purchased from Nihon SLC (Shizuoka, Japan). Animals were housed in an air-conditioned room (25°C) under a 12 hr light/dark cycle for at least 1 week before use. Food (MF diet; Oriental Yeast Co. Ltd., Tokyo, Japan) and water were provided ad libitum.

Preparation of rat liver mitochondria

Liver mitochondria were isolated by a previously described method (Schneider, 1948; Okuda et al., 2010) with modifications. After 24 hr fasting, male Wistar rats (6-8 weeks old) were anesthetized with pentobarbital and decapitated immediately, and the liver was removed and homogenized in ice-cold isolation buffer (250 mM sucrose, 10 mM HEPES-KOH, 0.5 mM EGTA) using a Potter homogenizer. The homogenate was centrifuged at 770 × g for 5 min at 4°C, and the resulting supernatant was further centrifuged at 9800 × g for 10 min. The pellet was resuspended in the same buffer and centrifuged at 2000 × g for 2 min and at 4500 × g for 8 min concurrently. The pellet was washed and resuspended in buffer (pH 7.4) containing 250 mM sucrose, 10 mM HEPES-KOH, and 0.3 mM EGTA, and centrifuged at 4500 × g for 10 min. The pellet was resuspended in the same buffer and centrifuged at 2000 × g for 2 min and at 4500 × g for 8 min concurrently. The pellet was washed and resuspended in buffer (pH 7.4) containing 250 mM sucrose, 10 mM HEPES-KOH, and used for further experiments.

Assessment of mitochondrial swelling

Mitochondrial swelling was assessed using a previous-
ly described method (Okuda et al., 2010). Mitochondria (0.5 mg protein/mL) were preincubated in swelling buffer (125 mM sucrose, 65 mM KCl, 5 mM succinate, 10 mM HEPES, 2.5 µM rotenone, pH 7.4) at 30°C. After preincubation for 2 min, the reaction was started by addition of troglitazone (10 µM final concentration). The absorbance at 540 nm was monitored. To examine the effects of CsA (an MPT inhibitor, Petronilli et al., 1994), BEL (a selective inhibitor of iPLA₂, Ong et al., 2015), bongkrekic acid (an inhibitor of ANT) and EGTA (a chelator), these substances were added before preincubation.

Evaluation of extra-mitochondrial Ca²⁺ concentration
Mitochondria (0.5 mg protein/mL) were incubated with drugs in the swelling assay system containing 50 µM arsenazo III. Extra-mitochondrial Ca²⁺ was assessed by recording changes in the differential absorbance of arsenazo III at wavelengths of 650 and 685 nm. Absolute Ca²⁺ concentration was calculated using standard solutions of Ca²⁺.

Measurement of CL and monolysocardiolipin (MLCL)
CL and monolysocardiolipin (MLCL) were measured using a liquid chromatography-mass spectrometry (LC-MS) system (amaZon SL; Bruker Daltonics Inc., Billerica, MA, USA). CL was extracted from mitochondria exposed to drugs for 40 min as described previously (Martens et al., 2015). For extraction of CL, 2.1 mL of chloroform/methanol (2:1, v:v) containing 0.05% butylated hydroxytoluene was applied to drug-exposed mitochondria (5 mg of protein). Lipid and aqueous phases were then separated by adding 400 µL of 0.01 M HCl, vortexing vigorously, and centrifuging at 1600 × g for 10 min. The lower phase was collected and dried at 40°C, and the pellet was dissolved in methanol and filtered using a 0.45 µm polytetrafluoroethylene filter. Samples (5 µL) were injected into a LC-MS system consisting of an amaZon SL ion-trap device connected to a Chromaster high-performance liquid chromatography (HPLC) system (Hitachi High-Tech, Tokyo, Japan). A YMC-Triart C18 column (150 × 2.0 mm internal diameter, 3 µm particle size; YMC Co., Ltd., Kyoto, Japan) was employed at 55°C. Two eluents were used: Eluent A (acetonitrile:water, 6:4, v:v) and eluent B (2-propanol:acetonitrile, 9:1, v:v). The gradient consisted of 40% B to 100% B over 25 min, 100% B for 20 min, and 40% B for 5 min, at a flow rate of 200 µL/min. As analytical targets, we selected three species of CL, (18:2)₄-CL, (18:2)₃(18:1)-CL, and (18:2)₂(18:1)₂-CL, and two species of MLCL, (18:2)₃-MLCL and (18:2)₂(18:1)-MLCL, that corresponded to the decomposed products of these CLs mentioned above. These compounds account for ~90% of total CL in rat liver mitochondria (Minkler and Hoppel, 2010). CL and MLCL were analyzed using Quant Analysis 2.2 (Bruker Daltonics Inc.).

Measurement of mitochondrial membrane potential
Mitochondrial membrane potential was measured as previously reported with some modification (Baracca et al., 2003). Briefly, mitochondria (1 mg/mL) was incubated with Rhodamine 123 (0.5 µM) in the buffer (125 mM sucrose, 65 mM KCl, 10 mM HEPES, 2.5 µM rotenone, pH 7.4) for 1 min and 5 mM succinate was added. After 5 min of incubation with drugs, the suspension was centrifuged at 8000 × g for 1 min and the fluorescence intensity (Ex:485 nm/Em:535 nm) of the supernatant was measured with FilterMax F5 (Molecular Devices, San Jose, CA, USA). Membrane potential was calculated with the Nernst equation;

\[ \Delta \phi = \frac{59 \log ([X]_{\text{in}}/ [X]_{\text{out}})}{z F} \]

In this equation, [X]_{\text{out}} was calculated using fluorescence intensity of the supernatant, while [X]_{\text{in}} was estimated from Rhodamine 123 uptake assuming distribution into a matrix space of 1.4 µL/mg protein (Kamo et al., 1979).

Statistical analyses
To demonstrate statistically significant differences between conditions, Student’s t-test was used for comparison of two means and one-way analysis of variance (ANOVA) with Bonferroni’s multiple comparisons test or Dunnett’s multiple comparisons test was used for multiple comparisons. A p-value < 0.05 was considered statistically significant. Statistical analyses were conducted using GraphPad Prism6 (GraphPad Software; San Diego, CA, USA).

RESULTS
Troglitazone stimulates the release of Ca²⁺ from mitochondria
Like endoplasmic reticulum (ER), mitochondria act as a store of intracellular Ca²⁺ to maintain a very low concentration of Ca²⁺ in the cytosol. Opening of the MPT pore, through which small molecules and ions pass freely, leads to the release of Ca²⁺ from mitochondria into the cytosol. Conversely, exogenously applied Ca²⁺ triggers...
MPT after it is taken up into matrix *in vitro*. Thus, Ca\(^{2+}\) and MPT are highly correlated with each other. Based on the assumption that Ca\(^{2+}\) status is related to troglitazone-induced MPT, we investigated the relationship between MPT induction and changes in extra-mitochondrial Ca\(^{2+}\) concentration by assessing mitochondrial swelling and Ca\(^{2+}\) concentration in swelling buffer before and after exposure to troglitazone.

First, we examined the dose-dependency of troglitazone-induced mitochondrial swelling. Fig. 1A shows that 10 µM troglitazone induced mitochondrial swelling but 5 µM did not. From this result, we set the concentration of troglitazone at 10 µM in following experiments. Next, as shown in Fig. 1B, troglitazone increased the concentration of extra-mitochondrial Ca\(^{2+}\) at 5 min to approximately 2 µM. This release was not suppressed by CsA (an MPT inhibitor) or BEL (a selective inhibitor of iPLA\(_2\)). On the other hand, mitochondrial swelling only started to occur at 20, not at 5 min, and was inhibited by CsA and BEL (Fig. 1C). Taken together, these results show that

**Fig. 1.** Relationship between changes in extra-mitochondrial Ca\(^{2+}\) concentration and MPT induction. (A) Mitochondrial swelling induced by various concentrations of troglitazone, (B) Troglitazone-induced changes in extra-mitochondrial Ca\(^{2+}\) concentration and (C) mitochondrial swelling in the presence or absence of an MPT inhibitor (CsA) or an iPLA\(_2\) inhibitor (BEL). Troglitazone (10 µM) or DMSO (0.1%) was added at t=0. CsA (2 µM) or BEL (10 µM) or bongkrekic acid (BkA, 20 µM) was added at 2 min before t=0. Ca\(^{2+}\) concentrations were calculated using a standard curve. In (A), the changes in absorbance in each condition were compared with “0 µM” using one-way ANOVA followed by Dunnett’s multiple comparisons test. Mean ± S.E. is shown for each time point. \(^*\)p < 0.05, \(^{**}\)p < 0.01 vs. “0 µM”. In (B) and (C) the changes in absorbance were compared between “Cont.” and “Tro”, “Tro” and “+CsA” or “+BEL” or “+BkA” using one-way ANOVA followed by Bonferroni’s multiple comparisons test. Mean ± S.E. is shown for each time point. \(^{*}\)p < 0.05, \(^{**}\)p < 0.01, \(^{***}\)p < 0.001 vs. “Cont.”, \(^\#\)p < 0.05, \(^{##}\)p < 0.01 vs. “Tro”. There were significant differences (p < 0.05) between “Tro” and “+BkA” after time=40 min, although the mark (\(^\#\)) of significant difference is not visible.
Ca²⁺ release precedes MPT induction and the involvement of iPLA₂. Moreover, troglitazone-induced MPT was inhibited by bongkrekic acid, an inhibitor of ANT (Fig. 1B), suggesting that ANT is involved in troglitazone-induced MPT. Furthermore, a second Ca²⁺ release occurred in troglitazone-treated mitochondria at 20 min (Fig. 1B), at the same time as MPT induction (Fig. 1C). Considering that the second Ca²⁺ release was CsA- and BEL-sensitive (Fig. 1B), it was most likely caused by MPT pore opening.

**Troglitazone-stimulated Ca²⁺ release is involved in MPT induction**

We showed above that Ca²⁺ is released from mitochondria before MPT induction and the involvement of iPLA₂. Although extra-mitochondrial Ca²⁺ reached only 2-3 µM at most, the concentration in the intermembrane space could have been much higher and sufficient to induce MPT. To confirm whether the released Ca²⁺ leads to MPT, we studied the effects of 0.5 mM EGTA (a Ca²⁺ chelating reagent) on troglitazone-induced mitochondrial swelling. As shown in Fig. 2, troglitazone did not induce swelling in the presence of EGTA. This result indicates that troglitazone-induced MPT is mediated by release of Ca²⁺ from mitochondria.

**Troglitazone enhances CL degradation**

It is believed that iPLA₂ activation leads to MPT induction (Kinsey et al., 2007). As a substrate of iPLA₂, we focused on the mitochondria-specific phospholipid CL, which supports mitochondrial morphology and various functions including the formation of respiratory supercomplexes (Zhang et al., 2002), retention of cytochrome c at the inner membrane (Nomura et al., 2000), and maintenance of ANT conformation (Nury et al., 2005). We therefore assessed CL decomposition by measuring the ratio of MLCL to CL in drug-exposed mitochondria. Troglitazone increased the ratio of MLCL to CL by 50%, and this increase was suppressed by co-exposure to BEL (Fig. 3). This result indicates that troglitazone causes decomposition of CL in an iPLA₂-dependent manner.

**Mild depolarization induces MPT**

It is reported that troglitazone depolarizes mitochondria (Masubuchi et al., 2006) and a drop in mitochondrial membrane potential leads to activation of mitochondrial iPLA₂ (Rauckhorst et al., 2014). To investigate whether depolarization was involved in MPT induction, we assessed the effect of troglitazone on mitochondrial membrane potential. As shown in Fig. 4A, a 5 min exposure to troglitazone decreased membrane potential by approximately 10%. Also, 100 nM CCCP (an uncoupling reagent) decreased membrane potential by the same extent.

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**Fig. 2.** The effect of a Ca²⁺ chelator on troglitazone-induced mitochondrial swelling. Troglitazone-induced mitochondrial swelling in the presence or absence of a Ca²⁺ chelator (EGTA). Mean ± S.E. is shown for each time point. Troglitazone (10 µM) or DMSO (0.1%) was added at t=0. EGTA (0.5 mM) was added 2 min before t=0. Decreases in absorbance were compared using one-way ANOVA followed by Bonferroni’s multiple comparisons test. Mean ± S.E. are shown for each time point. *p < 0.05, **p < 0.01, ***p < 0.001 vs. “Cont.”, p < 0.05, ##p < 0.01, ###p < 0.001 vs. “Tro”.

**Fig. 3.** Effects of troglitazone and the iPLA₂ inhibitor BEL on the monolysocardiolipin (MLCL):cardiolipin (CL) ratio. CL and MLCL were extracted from mitochondria after troglitazone-induced swelling. MLCL:CL ratios were compared using one-way ANOVA followed by paired Bonferroni’s multiple comparisons test (*p < 0.05, ***p < 0.001 one-sided test, Mean ± S.E., n=6).
(Fig. 4A) and also induced MPT (Fig. 4B). While depolarization with CCCP was concentration-dependent up to 400 nM, MPT was suppressed at CCCP concentrations above 200 nM. Considering that MPT and Ca\(^{2+}\) release induced by 100 nM CCCP were both BEL-sensitive (Fig. 4C and 4D), mild (approximately 10%) depolarization may be a key intermediate event in troglitazone-induced MPT via increase in extra-mitochondrial Ca\(^{2+}\) and iPLA\(_2\) activation.

**DISCUSSION**

It is known that troglitazone induces MPT in isolated mouse liver mitochondria at a concentration of 10 µM.
The mechanism of troglitazone-induced mitochondrial dysfunction

(Masubuchi et al., 2006). Although this is slightly higher than the clinical \( C_{\text{max}} \) of 6.4 \( \mu \text{M} \) (Loi et al., 1999), the concentration of troglitazone in liver tissue can be higher than in blood (10-12 fold in the case of rats) (Yokoi, 2010). Furthermore, troglitazone concentrates in human hepatocytes more than in rat hepatocytes \textit{in vitro} (Lee et al., 2010). Collecting them, it is conceivable that accumulated troglitazone in the liver exceeds 10 \( \mu \text{M} \) and induces MPT in hepatocytes in clinical situation. Regarding the mechanism, troglitazone-induced MPT appears to be mediated by iPLA\(_2\) activation (Okuda et al., 2010). However, in general, troglitazone-induced MPT has been studied in the presence of 10^{-5} M \( \text{Ca}^{2+} \), although the \( \text{Ca}^{2+} \) concentration in the cytosol is only 10^{-4} to 10^{-5} M under normal conditions. Thus, in the present work, to investigate the effects of troglitazone under physiological conditions, we studied MPT induction by troglitazone in the absence of added \( \text{Ca}^{2+} \). The results revealed that troglitazone still induced MPT in an iPLA\(_2\)-dependent manner under this condition (Fig. 1C), as described above. We then aimed to clarify the mechanism of troglitazone-induced MPT in more detail.

Exogenous \( \text{Ca}^{2+} \) addition induces MPT and activates mitochondrial iPLA\(_2\) (Moon et al., 2012). On the other hand, mitochondrial PLA\(_2\) is reported to be involved in functional regulation (Pfeiffer et al., 1979) and swelling (Waite et al., 1969; Pfeiffer et al., 1979) of mitochondria. Moreover, troglitazone is reported to decrease mitochondrial \( \text{Ca}^{2+} \) retention capacity (Masubuchi et al., 2006). Therefore, we hypothesized that extra-mitochondrial \( \text{Ca}^{2+} \) would be relevant to troglitazone-induced MPT, and monitored the extra-mitochondrial \( \text{Ca}^{2+} \) concentration. The results showed that troglitazone increased the extra-mitochondrial \( \text{Ca}^{2+} \) concentration prior to MPT induction, and the increase was not suppressed by BEL, a selective inhibitor of iPLA\(_2\) (Fig. 1B). Furthermore, the increase was not suppressed by CsA (Fig. 1B), demonstrating that the initial leakage of \( \text{Ca}^{2+} \) might not be attributable to classical MPT pore opening. It is important to note that chelating extra-mitochondrial \( \text{Ca}^{2+} \) by EGTA completely suppressed troglitazone-induced MPT (Fig. 2). These results suggested that troglitazone-induced \( \text{Ca}^{2+} \) release could be an important trigger for MPT induction. However, our interpretation that released \( \text{Ca}^{2+} \) might be involved in MPT induction is not consistent with previous reports claiming that \( \text{Ca}^{2+} \) in the mitochondrial matrix is involved in MPT pore opening (Hunter et al., 1976). This apparent discrepancy may be due to the difference in the \( \text{Ca}^{2+} \) concentration achievable between their studies and ours; they observed inhibition of MPT by preventing \( \text{Ca}^{2+} \) uptake into mitochondrial matrix in the presence of an excess amount of exogenously added \( \text{Ca}^{2+} \) (about 75 \( \mu \text{M} \)), whereas we conducted experiments without adding exogenous \( \text{Ca}^{2+} \) (which would be at most 3 \( \mu \text{M} \) even if all the endogenous \( \text{Ca}^{2+} \) is released from the mitochondrial matrix). Moreover, mitochondria have an external \( \text{Ca}^{2+} \) binding site and occupation of this site by a divalent metal ion inhibits MPT pore opening (Bernardi et al., 1993). Therefore, it is possible that MPT was inhibited by external \( \text{Ca}^{2+} \) binding to this site when \( \text{Ca}^{2+} \) uptake was blocked in the previous studies. On the other hand, based on our results, we think that stimulation of MPT pore opening by \( \text{Ca}^{2+} \) released at early times (5-10 min after troglitazone addition) would be stronger than inhibition of MPT pore opening by extra-mitochondrial \( \text{Ca}^{2+} \) because the inhibition is very weak (IC\(_{50} \sim 0.2 \text{mM} \text{Ca}^{2+}\)) (Hurst et al., 2017; Bernardi et al., 1993). Further investigations will be needed to confirm these interpretations.

As a candidate iPLA\(_2\) activator, we suggest mild depolarization because only a 10% decrease in membrane potential (induced by respiratory chain inhibition) is sufficient to activate iPLA\(_2\) by 2-fold (Rauckhorst et al., 2014). As shown in Fig. 4A, troglitazone decreased membrane potential by 10%, as did 100 nM CCCP, and BEL-sensitive mitochondrial swelling was induced by 100 nM CCCP (Fig. 4C). One study showed that 200 nM FCCP induces MPT in the presence of \( \text{Ca}^{2+} \) (Scorrano et al., 1997), while another study reported that depolarization suppresses MPT (Aronis et al., 2002). Collectively, these results suggest mild but not strong depolarization may be a trigger for iPLA\(_2\) activation and subsequent induction of MPT. However, 50 nM CCCP did not induce MPT despite that it decreased membrane potential by 10%. This result indicates that mild depolarization is not enough to induce MPT and troglitazone has some other undefined effects, which may support or augment the effect of depolarization.

The mechanism of troglitazone-induced \( \text{Ca}^{2+} \) release has not yet been clarified. However, considering that mitochondrial \( \text{Ca}^{2+} \) uptake by the mitochondrial calcium uniporter is voltage dependent (Gunter and Sheu, 2009), a decrease in membrane potential may be one possible mechanism. Supporting this, 100 nM CCCP induced CsA- and BEL-insensitive \( \text{Ca}^{2+} \) release from mitochondria (Fig. 4D).

In this study, we attempted to investigate the mechanism connecting iPLA\(_2\) activation and MPT induction. It has been reported that the conformation of ANT is regulated directly by CL, a mitochondria-specific phospholipid (Beyer and Klingenberg, 1985; Nury et al., 2005), and the affinity of CL for ANT is significantly decreased by CL oxidation or decomposition (Beyer and Nuscher, 1996),
suggesting that CL decomposition into MLCL and fatty acids is one candidate mechanism linking iPLA2 to MPT. Our results showing that iPLA2-mediated decomposition of three major CLs was enhanced by troglitazone exposure (Fig. 3) suggests three possible triggers for troglitazone-induced MPT; 1) a decrease in CL, 2) an increase in MLCL, and 3) an increase in free fatty acids from CL. The MPT pore is regulated by the ATP synthasome, including ATP synthase and ANT, and CL is involved in oligomerization of ATP synthase (Laage et al., 2015). Additionally, CL binding to ANT affects its conformation (Pestana et al., 2009). Taken together, these results suggest that a change in the conformation of ANT or in dimerization of the c-ring of ATP synthase caused by a decrease in CL may lead to MPT pore opening. CL also contributes to the formation of respiratory super-complexes (Zhang et al., 2002) and contact sites (Ardail et al., 1990). Thus, it is conceivable that, when the CL content is diminished, respiration may be uncoupled by the dissolution of super-complexes and diffusion of protons into the intermembrane space. Moreover, a decrease in CL could lead to the release of cytochrome c from mitochondria, since CL binds to cytochrome c on the outer side of the inner membrane. The involvement of MLCL in MPT has been poorly studied. By contrast, the involvement of free fatty acids in MPT has been well studied. For example, arachidonic acid liberated by iPLA2 is reported to induce MPT in Ca2+-treated mitochondria (Kinsey et al., 2007). Given that iPLA2 is an enzyme that removes oxidized acyl chains from CL (Liu et al., 2017) and remodels CL via the removal of saturated acyl chains (Zachman et al., 2010), another possibility is that differences in the characteristics of CL, such as its oxidative status, length, and degree of unsaturation of acyl chains, might determine sensitivity to troglitazone-induced MPT. However, considering that we could not detect significant increase in MLCL:CL ratio before swelling begins (data not shown), further investigation is needed to determine the mechanism connecting iPLA2 activation and MPT induction.

In conclusion, we suggest (1) CL decomposition caused by activation of iPLA2 and stimulation of Ca2+ release collaborate to trigger troglitazone-induced MPT, and (2) troglitazone decreases membrane potential by 10% and this may be a trigger for iPLA2 activation and Ca2+ release (Fig. 5). Although the importance of cardioplin and iPLA2 in MPT pore opening has been reported or suggested before, to the best of our knowledge, this is the first report indicating that a drug can trigger iPLA2 activation resulting in severe mitochondrial dysfunction including MPT. Further investigation is needed to uncover more

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**Fig. 5.** Scheme of this study. (A) The conformation of the MPT pore complex is maintained by CL. (B) Troglitazone removes CL from the complex by activating iPLA2, which might be mediated by mild depolarization, and induces Ca2+ release from the mitochondrial matrix. iPLA2 activation and stimulation of Ca2+ release would collaboratively trigger MPT pore opening via a change in the conformation of the MPT pore complex.
about the mechanism involved in troglitazone-induced death of hepatocytes and idiosyncratic liver injury.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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T. Sato et al.

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