The Mitochondrial Superoxide/Thioredoxin-2/Ask1 Signaling Pathway is Critically Involved in Troglitazone-Induced Cell Injury to Human Hepatocytes

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Although the mechanisms and susceptibility factors of troglitazone-associated idiosyncratic liver injury have not been elucidated, experimental evidence has identified oxidant stress and mitochondrial injury as a potential hazard *in vitro*. In search of upstream mediators of toxicity, we hypothesized that troglitazone-induced increased mitochondrial generation of superoxide might activate the thioredoxin-2 (Trx2)/apoptosis signal–regulating kinase 1 (Ask1) signaling pathway, leading to cell death, and that, hence, the mitochondrially targeted radical scavenger, mito-carboxy proxyl (CP), would prevent the increase in superoxide net levels and inhibit mitochondrial signaling and cell injury. Immortalized human hepatocytes (HC-04) were exposed to troglitazone (0–100μM), which caused concentration and time-dependent apoptosis after 12–24 h (ketoconazole-insensitive). We found that troglitazone rapidly dissipated the mitochondrial inner transmembrane potential ($\Delta\Psi_m$) and independently increased the net levels of mitochondrial superoxide by 5-fold. This was followed by a shift of the redox ratio of mitochondrial Trx2 toward the oxidized state and subsequent activation of Ask1. Cell injury, but not the decrease in $\Delta\Psi_m$, was prevented by cyclosporin A (3μM), indicating that mitochondrial permeabilization, but not membrane depolarization, was causally involved in cell death. Mito-CP not only decreased troglitazone-induced superoxide levels but also prevented Trx2 oxidation and activation of Ask1 and protected cells from toxic injury. These data indicate that troglitazone, but not its oxidative metabolite(s), produce intramitochondrial oxidant stress that activates the Trx2/Ask1 pathway, leading to mitochondrial permeabilization. Furthermore, the data support our concept that targeted delivery of an antioxidant to mitochondria can inhibit upstream signaling and protect from troglitazone-induced lethal cell injury.

Key Words: troglitazone; mitochondria; oxidative stress; idiosyncratic drug toxicity; thioredoxin-2; mito-CP.

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Troglitazone is a first-generation thiazolidinedione (TZD) insulin sensitizer that was withdrawn from the market due to an unacceptable risk of liver injury in patients. The unpredictable nature and the rare but severe hepatic adverse effects clearly indicate that these effects were based on drug idiosyncrasy. However, the reasons why very few patients developed liver injury, while the vast majority of recipients tolerated the drug well, has remained unknown, despite thorough evaluation of patient data and extensive experimental research aimed at elucidating the mechanisms of toxicity induced by troglitazone (reviewed by Chojkier, 2005; Masubuchi, 2006; Smith, 2003).

On the other hand, a clear hazard of troglitazone and other TZDs on well-defined *in vitro* models including hepatocyte cultures has been demonstrated, typically by using supratherapeutic exposure to the drug. In particular, compelling evidence has been accruing for a major role of mitochondria being both targets and mediators of troglitazone toxicity. This conclusion has been derived from the demonstration of adverse effects of troglitazone on the mitochondrial inner transmembrane potential (Masubuchi *et al.*, 2006; Tirmenstein *et al.*, 2002), inhibitory effects on complexes I through V of the electron transport chain (Brunmair *et al.*, 2004; Nadanaciva *et al.*, 2007; Soller *et al.*, 2007), and evidence for the subsequent induction of the mitochondrial permeability transition (mPT), leading to activation of proapoptotic members of the Bcl-2 protein family and cell death (Bae and Song, 2003; Masubuchi *et al.*, 2006; Soller *et al.*, 2007). Furthermore, we have recently demonstrated that prolonged administration of low (therapeutic) doses of troglitazone can induce mitochondrial damage associated with hepatic necrosis in a transgenic mouse model featuring heterozygous deficiency of the mitochondrial antioxidant superoxide dismutase-2 (Sod2) (Ong *et al.*, 2007), which sensitizes these mice to the mitochondria-targeting effects of troglitazone.

Although the exact mechanism of how troglitazone triggers mitochondrial injury is still unknown, several lines of evidence point to a pivotal role of reactive oxygen species (ROS) and oxidant stress (Jung *et al.*, 2007; Narayanan *et al.*, 2003;

Shishido *et al.*, 2003; Soller *et al.*, 2007). However, how exactly this oxidant stress may be linked to cell injury is not known. Our gap in understanding these mechanisms has been, at least in part, due to the fact that oxidant stress has traditionally been assessed in whole cells rather than in mitochondria. Furthermore, and importantly, it has not been convincingly demonstrated whether increased ROS production is the source or merely a consequence of cell injury provoked by troglitazone (Masubuchi, 2006).

With the advent of novel mitochondria-targeting probes that allow for selective delivery of a chemical into the mitochondrial matrix (Murphy and Smith, 2007), it has become possible to more specifically define intramitochondrial events, such as intramitochondrial ROS production and redox signaling. For example, by linking the superoxide-selective probe hydroethidine (HE) to the lipophilic cationic moiety, triphenylphosphonium (TPP), the resulting mito-HE allows for accurate estimation of superoxide levels in the mitochondrial matrix (Robinson *et al.*, 2006). Similarly, by linking TPP to an antioxidant, mitochondria-selective oxidant stress can be quenched (Murphy and Smith, 2007). The aim of this study was to use such fluorescent probes to specifically determine whether troglitazone induces mitochondrial oxidant stress in human hepatocytes and to explore the downstream signaling pathways and mechanisms that lead to lethal cell injury. We found that troglitazone greatly increased mitochondrial superoxide production, which activated the thioredoxin-2 (Trx2)/apoptosis signal–regulating kinase 1 (Ask1) pathway, triggering mitochondrial outer membrane permeabilization and cell death.

MATERIALS AND METHODS

Materials. Troglitazone was obtained from Cayman (Ann Arbor, MI). All other chemicals were purchased from Sigma (St Louis, MO), unless otherwise indicated, and of the highest degree of purity available.

Synthesis of mito-carboxy proxyl. Mito-carboxy proxyl (CP) was synthesized following a previously described method (Dhanasekaran *et al.*, 2005) with some modifications. Briefly, triphenylphosphine (13.1 g, 0.05 mol) and 11-bromo undecanol (12.6 g, 0.05 mol) were refluxed in benzene (150 ml) under a nitrogen atmosphere for 24 h, and then cooled to room temperature, after which the solvent was removed by decantation. The residual oil was dissolved in dichloromethane, and diethyl ether was added to precipitate the desired triphenylphosphine adduct of 11-bromo undecanol, which was removed by vacuum filtration. The process was repeated twice to give the adduct as a solid product. Thin-layer chromatography (chloroform:methanol 9:1) showed that the product was reasonably pure and it was used as such for reaction with the acid chloride of 3-chloroformyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (CP). The CP acid chloride was prepared in the following way: Pyridine (0.4 ml, 4.9 mmol) was added to a stirred suspension of CP (0.7 g, 3.7 mmol) in dry benzene (20 ml) and the reaction mixture cooled to 0°C in an ice bath, followed by drop-wise addition of thionyl chloride (0.3 ml, 5 mmol). After 30 min, the ice bath was removed and stirring continued until a clear solution was obtained. Dry ether (10 ml) was added to precipitate pyridinium hydrochloride, which was collected by filtration. The filtrate was concentrated *in vacuo* to give CP acid chloride as a yellow semisolid (0.7 g). The acid chloride was dissolved in dry chloroform (10 ml) and added drop wise to a stirred mixture of pyridine (0.35 ml) and the earlier-formed adduct (1.7 g, 3.3 mmol) in dry chloroform

(30 ml). The mixture was stirred at room temperature for 6 h and then worked up by successive washings with 1 N hydrochloric acid, distilled water, saturated NaHCO$_3$, and distilled water. After drying in anhydrous MgSO$_4$, the organic layer was concentrated to about 5 ml and added in a slow stream to dry ether (50 ml). The precipitated product was separated by careful decantation of the solvent, redissolved in chloroform and precipitated again with dry ether. This was repeated five to six times to give mito-CP as a viscous orange semisolid. Formation of product was verified by liquid chromatography-mass spectrometry. Mito-CP did not interfere with any of the biochemical assays used.

Culture of human immortalized HC-04 cells and drug exposure. HC-04 cells (Siam Life Science Ltd, Bangkok, Thailand) are metabolically competent immortalized human hepatocytes that readily respond to mitochondrial toxicants (Lim *et al.*, 2007). The cells were cultured in hepatocyte basal medium (HBM) supplemented with hepatocyte culture medium Bulletkit (Cambrex, East Rutherford, NJ) and 10% fetal bovine serum. Primaria flasks (Falcon) were precoated with 0.03 mg/ml of collagen solution, 0.01 mg/ml of fibronectin, and 0.01 mg/ml of bovine serum albumin in HBM for 2 h at 37°C before seeding of cells. The cells were maintained at 37°C in a humidified incubator with 5% CO$_2$ and passaged every 4–5 days at a ratio of 1:2. Prior to drug exposure, cells were plated and grown to subconfluency (~70–80%) in T75 flasks or monolayer in 96-well plates. The cells were washed with phosphate-buffered saline (PBS) and then treated with various concentrations of troglitazone for different periods of time. Troglitazone was dissolved in DMSO and then added to serum-free HBM (to avoid differential binding of troglitazone to plasma proteins). Other drugs were also predissolved in dimethyl sulfoxide and added to serum-free HBM. The final concentration of DMSO in each well was kept at 0.1% (v/v). Other chemicals including cyclosporin A (CsA) and mito-CP were dissolved in DMSO (final concentrations 0.1%) and added as indicated.

Assessment of cell injury. Release of lactate dehydrogenase (LDH) into the extracellular medium was selected as an indicator of cell injury and assayed as described earlier (Lim *et al.*, 2006). Apoptotic features of hepatocellular nuclei (chromatin condensation or fragmentation) were detected using the high-affinity nucleic acid stain, Hoechst 33342 (Invitrogen, Carlsbad, CA), which emits a blue fluorescence (461 nm) upon intercalation with DNA. About 200,000 cells were seeded on 0.01% poly-L-lysine-coated coverslips 24 h prior to drug exposure. At the respective time points, cells were washed twice with PBS, fixed with 4% paraformaldehyde for 20 min, air dried, and rinsed twice with PBS. Cells were then incubated with Hoechst 33342 (1:1000 in PBS) for 15 min in the dark. The coverslips were mounted onto glass slides with Histomount after rinsing with PBS and evaluated by fluorescence microscopy.

Measurement of mitochondrial transmembrane potential. JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide; Molecular Probes, Eugene, OR), a cationic dye that selectively accumulates within the mitochondrial matrix, was used to measure the mitochondrial membrane potential ($\Delta\Psi_m$) as described earlier (Lim *et al.*, 2006). The changes in fluorescence were recorded with a Safire 2 plate microreader (Tecan, Maennedorf, Switzerland).

Determination of mitochondrial superoxide production. To detect increased ROS production in mitochondria, we used the novel cell-permeable fluorogenic probe, mito-HE (MitoSOX Red; Invitrogen) as described earlier (Lim *et al.*, 2006).

Redox Western blotting for Trx2. Cell lysates were prepared by homogenizing cells in cell lysis buffer supplemented with Complete Mini protease inhibitor cocktail (Roche, Basel, Switzerland) by brief sonication. To detect both reduced and oxidized Trx2, the reduced Trx2 was immediately derivatized with 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) as described (Hansen *et al.*, 2006b). Cell debris was removed by centrifugation at 200 × g for 10 min at 4°C. Equal amounts of protein was mixed with Laemmli loading buffer under nonreducing conditions and heated for 2 min at 90°C. Protein was loaded into wells of a 1.5-mm thick 4% stacking gel (30% acrylamide, 1.5M Tris-HCl, pH 8.8, 10% sodium dodecyl sulfate

(SDS), 10% ammonium persulfate, and tetramethylethylenediamine dissolved in distilled water) and then separated through the resolving gel (similar composition with the exception of 0.5M Tris-HCl, pH 6.8, in place of 1.5M Tris-HCl, pH 8.8) by electrophoresis at 30 mA. Subsequent to electrophoresis, proteins were transferred onto nitrocellulose membranes in transfer buffer (25mM Tris-HCl, 0.2M glycine, 3mM SDS, and 20% methanol) at 100 V for 1 h. The membranes were blocked with 5% nonfat milk (in $1\times$ PBS-Tween-20) for 1 h and incubated overnight on an orbital shaker at 4°C with anti-Trx2 antibody (diluted 1:1000; Abcam, Cambridge, MA). Following $3\times$ washing with $1\times$ PBS-Tween-20 for 15 min each, the membranes were incubated with secondary horseradish peroxidase–conjugated anti-rabbit or anti-mouse antibody (Santa Cruz, CA) for 1 h with shaking. Using a chemiluminescence kit (Amersham Biosciences, Pittsburgh, PA), the membrane images were analyzed using a Gel Doc™ gel imaging system (BioRad, Hercules, CA).

Immunofluorescence detection of activated mitochondrial Ask1. Approximately 200,000 cells were seeded on poly-L-lysine-coated coverslips in six-well plates for at least 24 h. After treatment, the cells were washed twice with PBS, followed by incubation with 250nM MitoTracker Red CMXRos (Molecular Probes) for 30 min in serum-free medium. MitoTracker Red CMXRos is a red-fluorescent dye that specifically accumulates in the mitochondria and becomes fluorescent upon oxidation in the actively respiring cell and that was used as a mitochondrial marker. The cells were then fixed with 4% paraformaldehyde in serum-free medium for 20 min at room temperature and later permeabilized using 0.2% CHAPS in PBS for 2 min. The coverslips were blocked with 5% bovine serum albumin for 1 h and incubated overnight with anti-P-Ask1^{Thr845} (1:100; Cell Signaling Technology, Danvers, MA) at 4°C. After washing twice with PBS (+0.2% Tween-20), cells were probed with anti-mouse or anti-rabbit AlexaFluor 488-labeled secondary antibody (1:200 in 5% BSA) (Invitrogen) for another 1 h. Subsequent to four washings, coverslips were then mounted with Prolong anti-fade mounting reagent (Molecular Probes). Cells were examined with an Olympus FLOVIEW FV1000 confocal microscope using oil immersion at $\times$ 60 magnification.

Biochemical assays. The protein concentration of cell lysates and subcellular fractions was determined using the Bradford protein assay reagent (BioRad), using albumin as the standard protein.

Statistical analysis. For one experiment, the means of triplicate measurements (wells) was taken as one data point. All measurements were obtained from at least three independent experiments. The data obtained were expressed as mean ± SD and analyzed by ANOVA/Turkey-Kramer multiple comparison posttest (Instat; Graphpad, San Diego, CA). $p \leq 0.05$ was considered significant.

RESULTS

Troglitazone Induces Time- and Concentration-Dependent Cell Injury in Immortalized Human Hepatocytes

Exposure of HC-04 cells to troglitazone caused marked increases in LDH release, indicative of cell injury, that became significant at 12 h using concentrations of 75μM and higher (Fig. 1A). Thus, for most of the subsequent experiments, troglitazone concentrations of 100μM were used because any inhibitory effects would become readily detectable. Staining of exposed cells with Hoechst 33342 clearly revealed apoptotic nuclei (Fig. 1B), which confirms earlier data (Bae and Song, 2003; Soller *et al.*, 2007; Toyoda *et al.*, 2001). Because the major cytochrome P450 (CYP) form involved in bioactivation of troglitazone to reactive metabolites is CYP3A4 (He *et al.*, 2004), we used ketoconazole, a specific chemical inhibitor of this CYP isoform, to analyze effects of potential oxidative
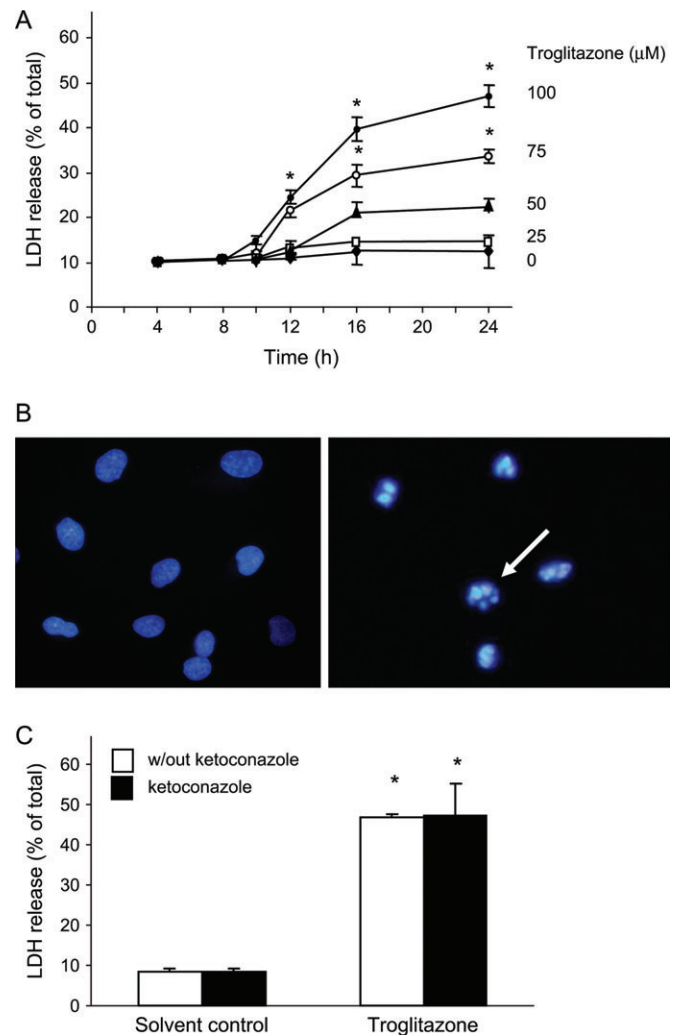

FIG. 1. Troglitazone induces lethal cell injury in HC-04 cells. (A) Concentration- and time-dependent release of LDH; (B) staining with Hoechst 33342 demonstrates apoptotic nuclei in cells (arrow) exposed to troglitazone (100μM) for 12 h; (C) lack of apparent effect of the CYP3A4 inhibitor ketoconazole (100μM) on the extent of cell injury in cells exposed to troglitazone (100μM) for 24 h. *$p \leq 0.05$ versus solvent control (0.1% DMSO).

metabolites on toxicity. As shown in Figure 3C, ketoconazole (100μM, a sufficiently high inhibitor concentration; Li and Jurima-Romet, 1997) did not have any apparent effects on LDH release induced by troglitazone, confirming in HC-04 cells that it is the parent compound rather than a reactive metabolite that is responsible for the toxicity (Tirmenstein *et al.*, 2002).

Troglitazone Causes Increases in Mitochondrial Superoxide Net Levels

Next, we confirmed and extended previous studies by exploring the effects of troglitazone on the mitochondrial inner transmembrane potential ($\Delta\Psi_m$). Troglitazone (50μM and greater) significantly decreased the $\Delta\Psi_m$ (Fig. 2A), which is
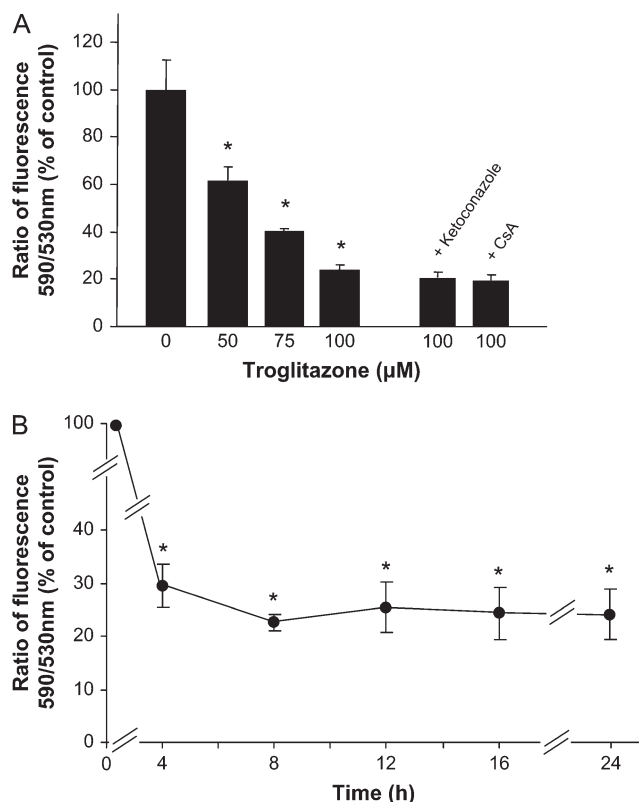
FIG. 2. Troglitazone decreases the mitochondrial inner transmembrane potential in HC-04 hepatocytes. (A) Cells were exposed to the indicated concentrations of troglitazone for 24 h in the presence or absence of ketoconazole ($100\mu M$) or CsA ($3\mu M$), washed, and loaded with JC-1. (B) Cells were incubated with $100\mu M$ troglitazone for the indicated time periods. The ratio of fluorescence at the emission wavelength of 590/530 nm was taken as a relative measure for the transmembrane potential (control = 100%, DMSO 0.1%). $*p \leq 0.05$ versus solvent control (A) or zero hours (B).

Fig. 3. Troglitazone causes increased mitochondrial net levels of superoxide anion that leads to mPT. (A) Concentration- and time-dependent increase in mito-HE fluorescence; (B) the mPT inhibitor, CsA ($3\mu M$) does not alter mito-HE fluorescence induced by exposure of cells to troglitazone ($100\mu M$) for 24 h; (C) CsA ($3\mu M$) inhibits troglitazone ($100\mu M$, 24 h)–induced lethal cell injury. $*p \leq 0.05$ versus solvent control (0.1% DMSO); $\#p \leq 0.05$ versus troglitazone alone.

in line with studies in isolated mitochondria (Masubuchi *et al.*, 2006) and rat hepatocytes (Haskins *et al.*, 2001). Importantly, neither ketoconazole nor CsA ($3\mu M$, a standard concentration to block the mPT in cultured hepatocytes; Kon *et al.*, 2004) prevented the ~80% decrease in $\Delta\Psi_m$ induced by $100\mu M$ troglitazone. This indicates that it is the parent compound, rather than a CYP3A4-dependent metabolite(s), that causes membrane depolarization. Furthermore, and importantly, these findings also confirm that the dissipation of the $\Delta\Psi_m$ is not only a consequence of the mPT but that the effects on the membrane potential must occur earlier, as a more upstream event. To further substantiate this, we have performed a time course of the loss of $\Delta\Psi_m$ following exposure to troglitazone (Fig. 2B). The data show that the depolarization became evident already at 4 h, i.e., clearly prior to the apparent onset of toxicity. This is also in accordance with earlier studies conducted in HepG2 cells (Bova *et al.*, 2005).

Most likely, the dramatic decrease in $\Delta\Psi_m$ could be caused either by the drug's uncoupling properties or by inhibition of the 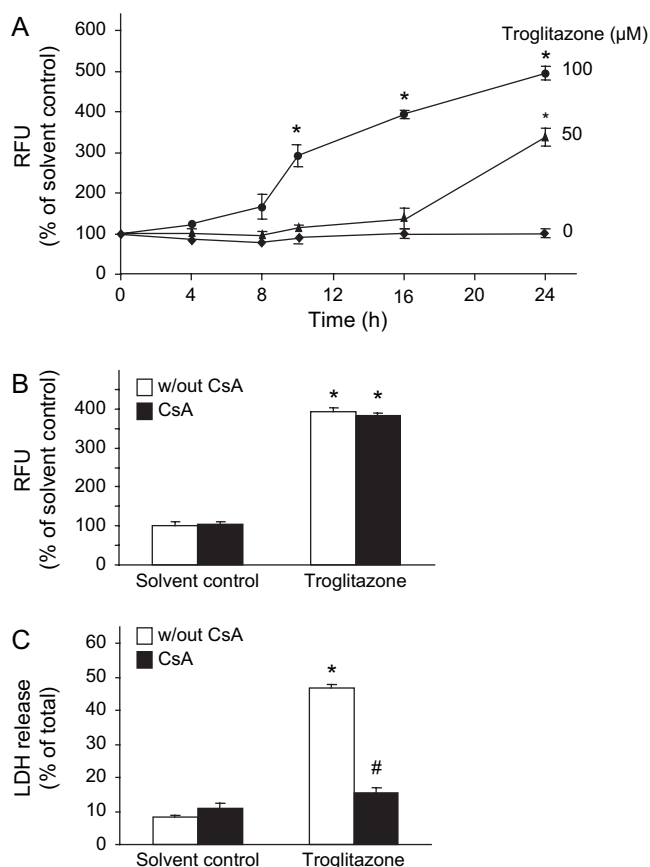electron transport chain, or both. There is no experimental evidence that troglitazone is a protonophoretic uncoupler of oxidative phosphorylation, but it has been demonstrated that troglitazone is a potent inhibitor of complexes I through V (Brunmair *et al.*, 2004; Nadanaciva *et al.*, 2007; Soller *et al.*, 2007). In addition, it was found that troglitazone can act as an uncoupler of state 2 (basal) respiration in isolated rat liver mitochondria, most likely through direct interactions with the inner mitochondrial membrane (Nadanaciva *et al.*, 2007). One of the consequences of the inhibition of complexes I, II, and III is an increase in mitochondrial ROS generation (Orrenius *et al.*, 2007). Therefore, we next determined whether troglitazone would specifically increase mitochondrial ROS using the mitochondria-selective probe, mito-HE. Figure 3A shows that troglitazone increased in a concentration-dependent manner the mitochondrial superoxide levels, becoming significant at 10 h ($100\mu M$) and reaching an almost 5-fold increase after 24 h. The oxidant stress preceded LDH release, indicating that the increased ROS levels were not simply a consequence of

mitochondrial injury and cell death. This conclusion was further corroborated by the findings that the mPT inhibitor CsA did not prevent this increase in ROS (Fig. 3B). Because CsA, in contrast to its effects on ROS formation, clearly inhibited cell injury due to mPT (Fig. 3C), we conclude that the enhanced mitochondrial superoxide formation is an upstream event (more proximal to the mPT), and we hypothesized that this mitochondrial oxidant stress is a causal event in the subsequent cell injury triggered by troglitazone.

Increased Levels of Superoxide Caused by Troglitazone Lead to Oxidation of Trx2 and Activation of Ask1

Increased levels of mitochondrial superoxide can disrupt the redox balance of the mitochondrial form of thioredoxin, Trx2 (Hansen *et al.*, 2006b). Therefore, we next determined whether troglitazone would alter the ratio of oxidized/reduced Trx2, which can be determined simultaneously in the same sample following derivatization of reduced Trx2 with AMS (Hansen *et al.*, 2006b). As compared to DMSO controls, troglitazone did not cause a shift in the Trx2 redox balance for up to 4 h; however, at 10 h there was a clear shift toward the more oxidized state, and at 16 h, there was no detectable reduced form left (Fig. 4), indicating that the marked intramitochondrial oxidant stress fully oxidized the Trx2 thiol groups and triggered the Trx2-dependent signaling cascade.

In its reduced form, Trx2 binds and thus inactivates Ask1 (Saitoh *et al.*, 1997). Following thiol oxidation of Trx2 through disulfide formation, Trx2 dissociates from Ask1, triggering the kinase activity of oligomerized Ask1 by phosphorylation of a critical threonine residue, which leads to activation of the mitogen-activated protein kinase (MAPK) cascade (Tobiume *et al.*, 2002) including c-Jun-NH_2-terminal protein kinase (JNK), which can ultimately lead to mPT. Therefore, we next assessed whether troglitazone was able to activate Ask1 in HC-04 cells. Immunofluorescence studies using a phospho-specific anti-Ask1[Thr845] antibody revealed that troglitazone, but not the

solvent alone (0.1% DMSO), activated Ask1, as judged by the strong fluorescence associated with the perinuclearly located mitochondria (Fig. 5). Simultaneous staining with the mitochondria-targeting probe MitoTracker Red revealed colocalization and confirmed that Ask1 was activated in mitochondria. Collectively, these findings suggest that the intramitochondrial oxidant stress is sufficiently high to activate the apoptosis-inducing MAPK cascade in hepatocytes.

Mito-CP Protects from Troglitazone-Induced Cell Injury by Reducing Intramitochondrial Oxidant Stress

If intramitochondrial superoxide formation, Trx2 oxidation, and Ask1 activation were indeed the cause for the subsequent signaling events leading to cell death, then inhibition of the proximal event would not only prevent Trx2 oxidation but also protect from cell injury. Toward this end, we concomitantly exposed hepatocytes to troglitazone (100μM) and the mitochondria-targeted antioxidant and radical scavenger, mito-CP (Dhanasekaran *et al.*, 2005). The results clearly revealed that mito-CP not only prevented the increase in net levels of mitochondrial superoxide (Fig. 6A) but also attenuated the oxidation of Trx2 (Fig. 6B). At 10 h, where (in the absence of mito-CP) troglitazone caused a marked shift toward the oxidized state of Trx2, there was still no change from the solvent controls, and at 16 h, where all Trx2 would be oxidized by troglitazone alone, there were still considerable amounts of reduced Trx2. Finally, and importantly, mito-CP protected cells from troglitazone-induced lethal cell injury (Fig. 6C), but it had no effect on the dissipation of $\Delta\Psi_m$ (data not shown). Taken together, the data indicate that the upstream events in the precipitation of troglitazone-induced lethal hepatocyte injury all occur in
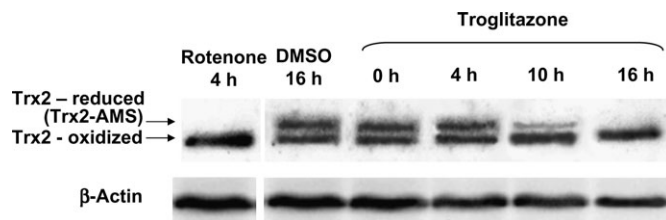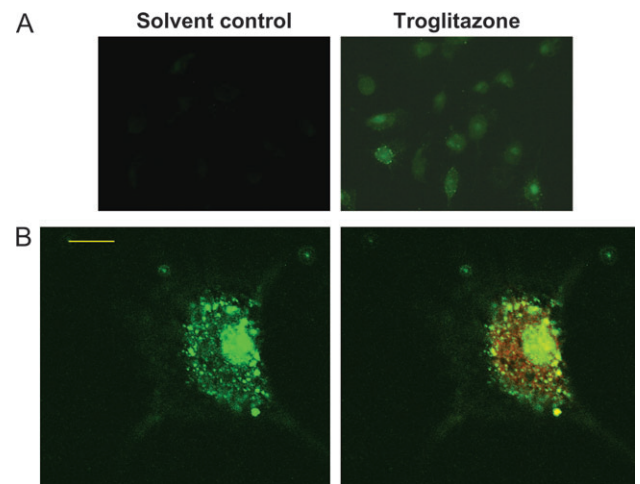

FIG. 4. Redox Western blot for both oxidized and reduced Trx2 after exposure of HC-04 cells to 100μM troglitazone or solvent (0.1% DMSO). At the indicated time points, cells were harvested and lysed, and the reduced Trx2 was immediately derivatized with AMS. Due to the higher molecular weight of AMS-Trx2, reduced and oxidized Trx2 can be resolved in the same lane with anti-Trx2 antibody. Rotenone (Rot, 50μM) served as positive control. β-Actin was included as loading control; because AMS interfered with protein quantification, the amounts of loaded protein were not corrected for protein content. This was considered of minor importance because the most relevant information is the ratio of band density of oxidized versus reduced Trx2 within the same lane.

FIG. 5. Ask1 immunofluorescence in HC-04 cells exposed to 100μM troglitazone or solvent (0.1% DMSO) for 10 h. Cells were permeabilized as described and incubated with an anti-P-Ask1[Thr845] antibody. (A) Troglitazone causes activation of Ask1 ($\times$ 200); (B) laser scanning confocal microscopy ($\times$ 400, oil immersion); left panel, anti-P-Ask1[Thr845] alone; right panel, overlay with MitoTracker Red staining; scale bar $=$ 10 μm.
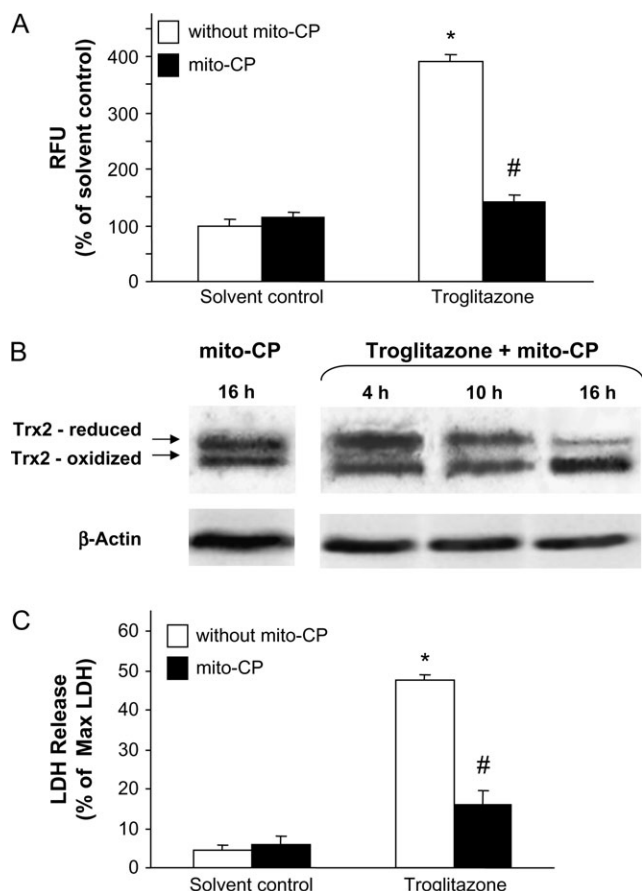
FIG. 6. Mito-CP attenuates mitochondrial oxidant stress, prevents Trx2 oxidation, and protects from cell injury induced by troglitazone (100μM). (A) Mito-HE fluorescence after exposure to combined troglitazone and mito-CP (50μM) for 24 h; (B) redox Western blot for both oxidized and reduced Trx2 after exposure of cells to 100μM troglitazone or solvent in the presence of absence of 50μM mito-CP. The results show that there was no apparent change in the Trx2 redox status for at least 10 h. (C) LDH release after exposure of cells to combined troglitazone and mito-CP (50μM) for 24 h. $*p \leq 0.05$ versus solvent control (0.1% DMSO); $\#P \leq 0.05$ versus troglitazone alone.

mitochondria and include increased superoxide formation leading to oxidation of Trx2 and activation of Ask1 (Fig. 7).

DISCUSSION

The purpose of this study was to explore the upstream mechanistic pathways in mitochondria that ultimately lead to the precipitation of lethal cell injury induced by troglitazone. We chose the immortalized human hepatocyte cell line HC-04 as a cellular model because these cells are metabolically competent and express human CYP3A4, the major CYP form involved in troglitazone bioactivation (He *et al*., 2004), at a level similar to primary human hepatocytes (Lim *et al*., 2007), and also because the mitochondrial signaling pathways leading to mitochondrial permeabilization are intact (Latchoumycandane
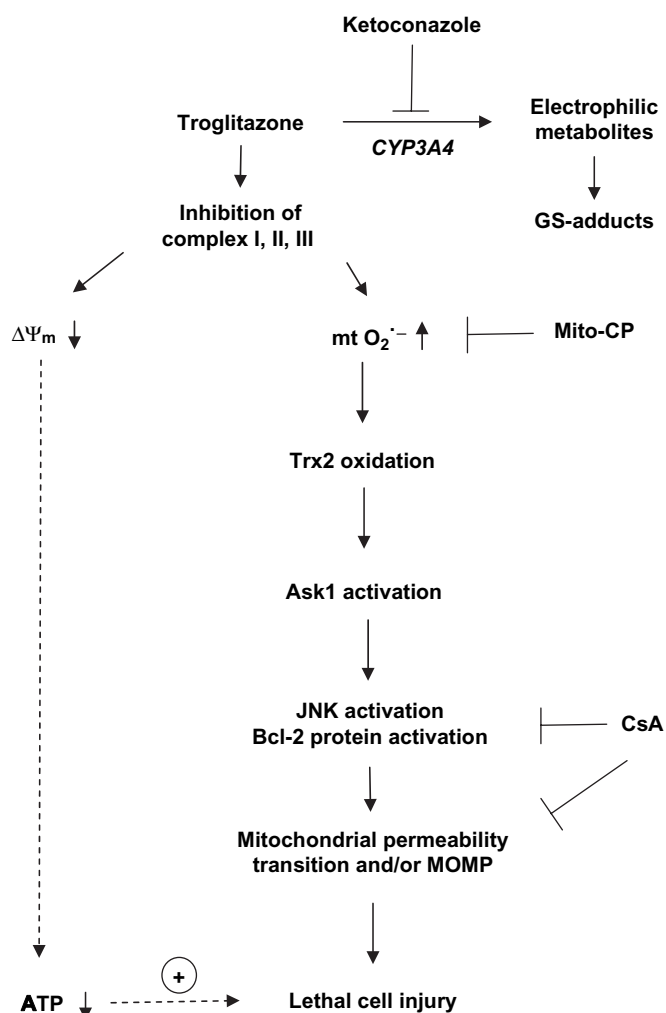

FIG. 7. Putative proximal pathways of troglitazone-induced cell injury in immortalized human hepatocytes (HC-04). Troglitazone is bioactivated by CYP3A4 to electrophilic intermediates that are trapped by glutathione, as inferred from the presence of glutathionyl (GS) adducts. On the other hand, the parent troglitazone interferes with mitochondrial function by inhibiting complexes I and II, which leads to dissipation of the inner transmembrane gradient and depletion of ATP and also to an increase in the net levels of mitochondrial superoxide. The oxidant stress posed by increased $O_2^{\cdot-}$ shifts the redox balance of Trx2 to the more oxidized state and activates Ask1, which in turn phosphorylates JNK and activates members of the Bcl-2 protein family. Due to the energy depletion and increased oxidant stress in the presence of mPT, the cells primarily undergo necrosis. Upstream inhibition of increased superoxide net production with the mitochondrially targeted nitroxide mito-CP not only decreases oxidant stress but also inhibits all downstream events and protects from troglitazone-induced cell death.

et al., 2006; Siu *et al*., unpublished data). We found that troglitazone, but not its oxidative metabolite(s), increased the steady-state levels of superoxide specifically in the mitochondrial matrix and that this mitochondrial oxidant stress led to oxidation of Trx2 and subsequent activation of Ask1 in hepatocytes. The Trx2/Ask1 pathway is a major upstream initiator of the MAPK pathway including JNK, which

invariably leads to the mPT and cell death (Hansen *et al.*, 2006a; Imoto *et al.*, 2006; Tobiume *et al.*, 2002; Zhang *et al.*, 2004). We conclude that the mitochondrial activation of the Trx2/Ask1 axis is a critical early event in troglitazone-induced hepatocyte injury.

These conclusions were based on a number of observations. Firstly, CsA, a chemical inhibitor of the mPT and certain upstream mediators including JNK (Matsuda and Koyasu, 2000; Siu *et al.*, unpublished data), protected from cell injury but did not prevent the troglitazone-induced increases in net ROS levels or the dissipation of the $\Delta\Psi$m. Thus, mitochondrial oxidant stress is not simply a consequence of mPT. These data are in contrast to an earlier study in Novikoff rat hepatoma cells, where intracellular superoxide production was inhibited by CsA (Narayanan *et al.*, 2003) and in which the authors concluded that oxidant stress is secondary to the opening of the mPT pore. However, in this latter study, ROS were measured with the probe HE, which does not selectively target mitochondria and which, therefore, rather gives an estimate of a general cellular oxidant stress.

Second, and importantly, the novel stable nitroxide and antioxidant, mito-CP, not only attenuated the increased ROS levels in mitochondria but also inhibited all downstream activation pathways and protected from lethal cell injury, implicating a causal role of superoxide in triggering cell injury. Mito-CP is specifically targeted to mitochondria due to the chemical linking of the nitroxide moiety to the lipophilic and cationic TPP moiety, which causes accumulation in the mitochondrial matrix by up to 500-fold as compared to the extracellular medium (Dhanasekaran *et al.*, 2005; Murphy and Smith, 2007). Mito-CP is not only a radical scavenger but possesses also superoxide dismutase activity and thus effectively removes superoxide from the site of where it is generated. Here, we demonstrate that mito-CP prevents troglitazone-induced cell injury, which is mediated through a mechanism involving Trx2 oxidation and activation of Ask1. This is typical for compounds that induce intramitochondrial oxidant stress through inhibition of complex I of the electron transport chain, e.g., rotenone (Hansen *et al.*, 2006b). Troglitazone has indeed been shown to inhibit complex I (and complex II as well) in Jurkat T cells (Soller *et al.*, 2007) and in sonicated rat liver (Brunmair *et al.*, 2004), as well as to significantly decrease the activity of complexes I–V after immunocapturing (Nadanaciva *et al.*, 2007). This is not unique for troglitazone but holds true for other TZDs, e.g., complex I inhibition has been demonstrated for rosiglitazone in skeletal muscle and rat liver homogenates (Brunmair *et al.*, 2004) and for ciglitazone in a leukemia cell line (Scatena *et al.*, 2004).

Apart from inhibition of the mitochondrial electron transport chain, which invariably results in increased oxidant stress, other mechanisms possibly involved in troglitazone toxicity cannot be excluded. For example, troglitazone can be oxidized to a phenol-*O* radical by peroxidase/H_2O_2. This induces glutathione (GSH) oxidation and cell injury (Tafazoli *et al.*,

2005); however, it is not known whether this reaction could also occur in intact cells or mitochondria. Nevertheless, it is intriguing to speculate that such novel drug radicals could be formed, especially in view of recent reports demonstrating that certain enzymes in mitochondria do exhibit peroxidase activity following activation, including SOD1 and cytochrome *c* (Basova *et al.*, 2007).

It has been previously described that troglitazone induces oxidant stress in nonhepatic cells and in hepatoma cell lines (which are not competent to metabolize troglitazone through CYP). For example, troglitazone caused apoptosis in HepG2 cells (Bae and Song, 2003) and in an osteoblast cell line (Jung *et al.*, 2007). The authors had concluded that troglitazone-induced ROS were not causally involved in toxicity because the antioxidant, *N*-acetylcysteine (NAC) did not protect from cell injury (Jung *et al.*, 2007). However, in contrast to the present study, these previous reports measured the cellular redox changes and did not specifically look at mitochondrial events, which could be a possible explanation for the inability of NAC to prevent or revert the oxidative changes in mitochondria. Thus, our findings are novel because for the first time we specifically addressed mitochondrial pathways rather than overall intracellular changes.

Trx2 plays a key role in mitochondria as a regulator of ROS-dependent stress signaling because of its role that is independent from GSH and distinct from the cytosolic form, Trx1. In contrast to GSH, where there is an exchange between the cytosolic and mitochondrial pools, Trx is not exchanged between these two subcellular compartments. Trx2 oxidation therefore closely reflects intramitochondrial oxidant stress that can trigger the activation (phosphorylation) of Ask1 and, further downstream, activation of the MAPK pathways. Indeed, troglitazone has been implicated in activating JNK and p38, which was followed by increased levels of truncated Bid, Bax, and Bad, and release of cytochrome *c* (Bae and Song, 2003). That JNK is critically involved in mediating mitochondrial permeabilization has been convincingly demonstrated by the use of the JNK inhibitor SP600125, which blocked troglitazone-induced cell death (Bae and Song, 2003). Other studies have provided further evidence for the involvement of the MAPK pathways; troglitazone-induced cell death was associated with downregulation of extracellular signal-regulated kinase (ERK) and upregulation of p38 (Jung *et al.*, 2007). We have identified for the first time the very upstream mediators that link ROS production with the activation of the MAPK pathways.

A still unresolved question is how these and earlier findings (that all describe a toxic hazard at relatively high and clearly supratherapeutic drug concentrations *in vitro*) can be reconciled with the rare but significant cases of idiosyncratic liver injury in patients who were prescribed troglitazone. The systemic exposure in patients was clearly lower, although troglitazone has been shown to accumulate in the liver reaching liver/plasma ratios of 15–20 (Kawai *et al.*, 1997). Typically, the

insult became clinically manifest only after a long lag time (months). This is compatible with gradual accumulation of changes in mitochondria in particularly susceptible individuals who may have featured a genetic or acquired deficiency in antioxidant defense systems or other mitochondrial abnormalities (Boelsterli and Lim, 2007). Clinical evidence in support of this hypothesis is sparse; one report found a correlation between susceptibility to troglitazone liver injury and a combined GST-T1 and GST-M1 null genotype, which could be a possible genetic susceptibility factor for troglitazone (Watanabe *et al.*, 2003). Further evidence for this concept stems from a study with heterozygous $Sod2^{+/-}$ mice (a murine model for clinically silent mitochondrial abnormalities) in which therapeutic doses of troglitazone administered daily for 4 weeks were able to induce mitochondrial injury and hepatocellular necrosis in these mice but not in normal healthy mice (Ong *et al.*, 2007). Clearly, more studies are needed to explain the susceptibility factors in patients; however, a detailed knowledge of the underlying molecular mechanisms and signaling pathways is crucial in understanding the pathomechanisms of troglitazone-induced cell injury and defining possible abnormalities.

In conclusion, we have established that in immortalized human hepatocytes, troglitazone caused a net increase in superoxide anion production specifically in mitochondria and that this enhanced intramitochondrial oxidant stress caused a shift in the redox state of Trx2 toward the oxidized state, which in turn led to activation of Ask1-dependent cell death–signaling pathways. By using a novel mitochondrially targeted radical scavenger, we have also demonstrated that targeted delivery of an antioxidant to mitochondria can rescue hepatocytes from troglitazone-induced lethal cell injury. Upstream inhibition of signaling events, prior to their branching out into different directions, may therefore become important and bear potential clinical applications.

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