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Troglitazone-Induced Hepatic Necrosis in an Animal Model of Silent Genetic Mitochondrial Abnormalities

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Troglitazone, a first-generation thiazolidinedione antidiabetic drug, was withdrawn from the market due to an unacceptable risk of idiosyncratic hepatotoxicity. Troglitazone does not cause hepatotoxicity in normal healthy rodents, but it produces mitochondrial injury *in vitro* at high concentrations. The aim of this study was to explore whether genetic mitochondrial abnormalities might sensitize mice to hepatic adverse effects of troglitazone. We used heterozygous superoxide dismutase 2 (*Sod2*$^{+/-}$) mice as a model of clinically silent mitochondrial stress. Troglitazone was daily administered for 4 weeks (0, 10 or 30 mg/kg/day, ip). We found that troglitazone caused overt liver injury in the high-dose group, manifested by increased serum alanine aminotransferase activity (> twofold) and midzonal areas of hepatic necrosis, in *Sod2*$^{+/-}$ but not in wild-type mice. No signs of hepatotoxicity were apparent at 2 weeks of treatment. Hepatic mitochondria isolated from troglitazone-treated mice exhibited decreased activities of aconitase (by 45%) and complex I (by 46%) and increased (by 58%) protein carbonyls, indicative of enhanced mitochondrial oxidant stress. This was paralleled by compensatory increases in mitochondrial glutathione levels. Finally, in hepatocytes isolated from untreated *Sod2*$^{+/-}$, but not wild-type mice, troglitazone caused a concentration-dependent increase in superoxide anion levels as demonstrated with a selective mitochondria-targeting fluorescent probe. In conclusion, prolonged administration of troglitazone can superimpose oxidant stress, potentiate mitochondrial damage, and induce delayed hepatic necrosis in mice with genetically compromised mitochondrial function. These data are consistent with our hypothesis that inherited or acquired mitochondrial abnormalities may be one of the contributing determinants of susceptibility to troglitazone-induced idiosyncratic liver injury.

Key Words: **drug-induced liver injury (DILI); idiosyncratic drug toxicity; oxidative stress; mitochondria; determinants of susceptibility; hepatotoxicity; superoxide dismutase.**

Troglitazone (Rezulin) is a first-generation thiazolidinedione insulin sensitizer and antidiabetic drug that was withdrawn after successful launching due to an unacceptable high risk of serious idiosyncratic drug-induced liver injury (DILI) (Graham *et al.*, 2002; Watkins and Whitcomb, 1998). Nonclinical safety studies had not revealed any hepatotoxicity in experimental animals including monkeys, although these latter exhibit a similar metabolite profile as humans (Herman *et al.*, 2002; Rothwell *et al.*, 2002). Despite considerable efforts, the determinants of individual patient susceptibility to troglitazone hepatotoxicity are currently not known (Chojkier, 2005; Smith, 2003).

A hallmark of troglitazone-induced hepatic injury was the delayed onset (months) of liver disease, which then could abruptly develop into hepatic failure, and this was linked to an increased risk with continued use (Graham *et al.*, 2002). This raises the possibility of a threshold effect typical for an accumulating but clinically silent insult, reminiscent of gradual mitochondrial injury induced by mitochondria-targeting drugs (DiMauro and Schon, 2003). Indeed, in experimental systems, troglitazone has been shown to adversely affect mitochondrial function, in particular by decreasing the mitochondrial membrane potential ($\Delta\Psi_m$), uncoupling oxidative phosphorylation, and inducing the membrane permeability transition (mPT) (Haskins *et al.*, 2001; Masubuchi *et al.*, 2006; Tirmenstein *et al.*, 2002). Furthermore, *in vitro* exposure of hepatocytes or liver-derived cell lines to high concentrations of troglitazone caused apoptotic cell death (Toyoda *et al.*, 2001; Yamamoto *et al.*, 2001).

However, if uncoupling and/or mPT induction in mitochondria were indeed a relevant mechanism of troglitazone toxicity, then this hazard alone cannot readily explain the low incidence and delayed onset of DILI in patients because the overwhelming majority of patients did not develop any hepatic adverse reactions to this drug. In line with this, it has been impossible to induce overt liver toxicity in normal healthy rodents even with high doses of troglitazone. We have therefore hypothesized that underlying mitochondrial abnormalities may predispose individuals to the mitochondria-damaging effects of mitochondria-targeting drugs (Boelsterli, 2003; Ong *et al.*, 2006). In fact, an increasing number of genetic mitochondrial abnormalities have

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become recognized (DiMauro and Schon, 2003; Pulkes and Hanna, 2001), some of which with well-known phenotypic manifestations. Alternatively, normal mitochondria can also gradually accumulate damage from acquired conditions, including oxidative damage to mitochondrial (mt) DNA from aging, disease conditions as diabetes, or drugs (Pessayre *et al.*, 2003; Suzuki *et al.*, 1999), and this could occur in a much more discreet way and remain clinically undetected. However, the response of such genetically altered mitochondria to hepatotoxic drugs *in vivo* has not been experimentally addressed.

In this study, we sought to determine whether mice with an underlying silent genetic mitochondrial damage were more sensitive to troglitazone than normal mice. To this end, we used the manganese superoxide dismutase (*Sod2*) heterozygous mouse model (Lebovitz *et al.*, 1996). Besides the primarily cytosolic SOD1, which is also present in the intermembrane space of mitochondria, SOD2 is the major mitochondrial SOD form and a crucial antioxidant. While the homozygous negative genotype is lethal postnatally, heterozygous ($Sod2^{+/-}$) mice appear normal and cannot be distinguished from their wild-type littermates. They also exhibit normal growth curves and breed well (Van Remmen *et al.*, 1999). However, because they express only approximately 50% of wild-type Sod2 activity, $Sod2^{+/-}$ mice acquire a number of changes in various organs that are all due to the accumulating consequences of excessive mitochondrial oxidative stress. For example, in liver, a number of distinct alterations have been characterized that become gradually worse with increasing age of the mice. These markers of oxidative injury include decreased mitochondrial membrane potential ($\Delta\Psi_m$), significant decreases in complex I activities and state 3 respiration, increased levels of 8-hydroxydeoxyguanosine, and an increased propensity for the induction of the mitochondrial mPT (Kokoszka *et al.*, 2001; Van Remmen *et al.*, 2003; Williams *et al.*, 1998). We have recently demonstrated that heterozygous *Sod2* knockout mice were sensitive to the mitochondria-damaging effects of prolonged administration of the nitroaromatic drug nimesulide *in vivo* and that they developed mitochondrial injury and an increased number of apoptotic hepatocytes (Ong *et al.*, 2006). We therefore hypothesized that $Sod2^{+/-}$ mice would be sensitized to the mitochondria-targeting effects of troglitazone, and that these cumulative effects would be translated into overt liver injury.

The aim of this study was to analyze the toxic response in the liver of $Sod2^{+/-}$ mice treated for 28 days with troglitazone at doses comparable to previously used dosage in humans. We found that troglitazone not only caused clear mitochondrial oxidative damage in such genetically compromised mice but also induced delayed hepatic necrosis.

MATERIALS AND METHODS

Sod2 knockout mice. All protocols involving animals were in compliance with the Institutional Animal Care and Use Committee and in accordance with the guidelines of the National Advisory Committee for Laboratory Animal Care and Research. Heterozygous $Sod2^{tm1Leb}/J$ mice (breeding pairs), congenic in the C57BL/6 background, were obtained from The Jackson Laboratory (Bar Harbor, ME). A breeding colony was established by crossing male $Sod2^{+/-}$ with female $Sod^{+/+}$ mice. The F_1 littermates were genotyped and subsequently used for the experiments. The mice were kept under specified pathogen-free conditions under controlled environmental conditions (22 ± 2°C, 75 ± 5% relative humidity, 12/12 h dark/light cycle) and had free access to standard rodent chow (Specialty Feeds Pte Ltd, Glen Forrest, Western Australia) and water *ad libitum*. All animals were 16–21 weeks old at the start of drug treatment.

Genotyping of* $Sod2^{+/-}$ *mice. Genotyping of all mice was performed prior to weaning. The assays were performed by polymerase chain reaction (PCR) analysis according to the protocol provided by The Jackson Laboratory. Briefly, the DNA was extracted from a < 4-mm tailpiece using a DNA tissue kit (Promega, Research Instruments, Singapore). PCR was performed with a Taq polymerase kit (Promega). The primers for the hypoxanthine guanine phosphoribosyl transferase minigene (240 bp) were 5′-TgT TCT CCT CTT CCT CAT CTC C-3′, oIMR0781, and 5′-ACC CTT TCC AAA TCC TCA gC-3′, oIMR0782, synthesized by Research Biolabs (Singapore). As internal standard, the *Sod1* gene was used; primers for the 123 bp product were 5′-TgA ACC AgT TgT gTT gTC Agg-3′ and 5′-TCC ATC ACT ggT CAC Tag CC-3′.

Drug administration and experimental design. Troglitazone ((±)-5-[4-(6-hydroxy-2,5,7,8-tetramethylchroman-2-ylmethoxy)benzyl]-thioazolidine-2,4-dione; from Cayman Chemical, Ann Arbor, MI) was dissolved in Solutol HS-15 (9% in phosphate-buffered saline [PBS]), a nontoxic solvent used for parenteral administration of water-insoluble compounds. Solutol HS-15 (BASF, Ludwigshafen, Germany) is composed of polyglycol mono- and di-esters of 12-hydroxystearic acid and 30% free polyethylene glycol. Troglitazone (10 or 30 mg/kg bw) or vehicle (10 μl/g bw) was injected ip daily at 8.00 A.M. for 14 or 28 days. The high troglitazone dose used here in mice is similar to the previously used therapeutic human dose (200 mg) (Loi *et al.*, 1999) if corrected for interspecies dose scaling factors (Kirman *et al.*, 2005). Systemic exposure (AUC) was also comparable to humans, and the plasma levels (C_{max}) in mice after a single dose of 30 mg/kg troglitazone were approximately 20μM (New, Saha, Ong, Boelsterli, and Chan, unpublished data). The ip route was selected in an attempt to target the drug to the liver and to guarantee maximal absorption.

Wild-type ($Sod2^{+/+}$) mice were assigned to two groups (vehicle and high-dose troglitazone; $n = 5$). Mutant $Sod2^{+/-}$ mice were randomly assigned to three groups (vehicle, low-dose, and high-dose troglitazone; $n = 7$, 7, and 14, respectively). After 4 weeks of treatment, the fed mice were anesthetized with pentobarbital (60 mg/kg). Blood was collected by cardiac puncture, and serum was prepared. The liver was quickly excised and weighed. While one portion was used for histopathology, the rest was minced and homogenized on ice and immediately used to prepare mitochondrial and cytosolic fractions. In a separate experiment, the time course of troglitazone hepatotoxicity was assessed. To this end, $Sod2^{+/-}$ mice were assigned to two groups (vehicle and high-dose troglitazone; $n = 4$), and the degree of hepatic changes was determined after 2 weeks.

Isolation of hepatic mitochondria and cytosol. Liver mitochondria and cytosol were isolated according to standard methods (Ong *et al.*, 2006). Protein content was determined with the Bradford reaction using albumin as reference protein. Both mitochondrial and cytosolic fractions were immediately snap frozen in liquid nitrogen and kept at − 80°C until used for analysis.

Serum ALT and liver histopathology. Serum activity of ALT was determined spectrophotometrically using a test kit (Catachem, Bridgeport, CT). For histopathological analysis, small pieces of liver were fixed in 10% phosphate-buffered formalin. The fixed tissues were subsequently processed with an automatic tissue processor (Leica TP 1020, Wetzler, Germany) and embedded in paraffin blocks. Tissue sections (5 μm) were stained with hematoxylin-eosin (H&E) and analyzed by light microscopy.

TUNEL staining, aconitase activity assay, determination of protein carbonyl content. All three assays were performed as described earlier (Ong *et al.*, 2006). TUNEL-positive (dark brown) nuclei were considered to reflect apoptotic changes, while areas of cells in which the cytoplasm was also stained were indicative of oncotic necrosis.

Determination of NADH:ubiquinone oxidoreductase (complex I) activity. Complex I activity was determined as described (Estornell *et al.*, 1993). Briefly, mitochondria (37 μg/ml, final protein concentration) was added to a freshly prepared reaction mixture consisting of 50mM KCl, 10mM Tris/HCl (pH 7.4), 1mM EDTA, 75μM NaN_3 (to block complex IV) and 1μM antimycin A (to block complex III). The mixture was incubated for 5 min before adding 50μM CoQ1 and 75μM NADH. Complex I activity was measured by monitoring the decrease in NADH concentrations (changes in absorbance at 340 and 380 nm) over 10 min. Rotenone (1μM) was used as a positive control.

Determination of glutathione. Glutathione (GSH) was determined in isolated mitochondria using monochlorobimane (mBCl) as fluorogen. mBCl features a high selectivity for GSH and is conjugated to GSH by a glutathione-*S*-transferase (GST)–catalyzed reaction. Samples were deproteinated with trichloroacetic acid (5%), centrifuged, and the supernatants incubated with 100μM mBCl and 1 U/ml GST in PBS, pH 7.4, for 30 min at 37°C. The fluorescence of the GS adducts was determined at 385/478 nm, and GSH levels were calculated from a standard curve.

Western immunoblotting. Equal amounts of denatured mitochondrial protein were loaded per lane, separated on a 15% SDS-PAGE gel under reducing conditions, and subsequently transferred to nitrocellulose membranes. The membranes were blocked in 5% nonfat dry milk prepared with 0.05% PBS-Tween 20 for 1 h at 25°C. Anti-SOD2 (1:1000) was used as primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-complex II 30 kDa subunit (Molecular Probes, Eugene, OR) was used as loading control (1:1000). The protein bands were visualized by enhanced chemiluminescence (Amersham Biosciences, Singapore) after incubation with HRP-conjugated secondary antibody (1:100,000).

Isolation and culture of mouse hepatocytes. Hepatocytes were isolated by the standard two-step collagenase retrograde perfusion technique. Wild-type or *Sod2*$^{+/-}$ mice were anesthetized with pentobarbital (60 mg/kg, ip) and laparotomy was performed. The liver was perfused *in situ* through the inferior vena cava using calcium-free Earle's Balanced Salt Solution (Invitrogen, Singapore), followed by Liver Digest Medium (Gibco, Carlsbad, CA). Perfusate flow was adjusted to 7 ml/min, exiting via the severed portal vein. The liver was then removed and gently scraped with a sterile scalpel to release the hepatocytes. The cell suspension was then filtered through a 60-μm pore size nylon membrane (Fisher, Pittsburgh, PA), washed twice for 3 min each by centrifugation at 50 × g at 4°C and finally resuspended in Hepatocyte Wash Medium (Gibco). Typically 24–36 × 10^6 viable cells were obtained from one mouse liver. Viability, assessed by trypan blue exclusion, was > 85%. The cells were seeded in collagen-coated six-well plates (Becton Dickinson, Singapore) at a density of 1 million viable cells per well. After an attachment period of 3 h at 37°C in 5% CO_2/95% air, the medium was replaced by Hepatocyte Maintenance Medium (HMM, Cambrex, Walkersville, MD), supplemented with antibiotics and hormones (HCM Single Quotes, Cambrex). After an overnight preculture period (18–20 h), the medium was replaced with HMM (without Single Quotes), and troglitazone was added from a stock solution in DMSO. To avoid any inhibitory effects on cytochrome P450 forms, the DMSO concentrations were kept at 0.1% (vol/vol).

Determination of mitochondrial superoxide anion. To selectively detect superoxide production in mitochondria of intact cells, the mitochondria-targeting fluorescent probe MitoSOX Red (Molecular Probes) was used. MitoSOX Red is a novel derivative of dihydroethidine coupled to the mitochondria-targeting triphenylphosphonium cation moiety through a hexyl linker. Hepatocytes were loaded with MitoSOX Red (5μM) at 37°C for 10 min, washed with PBS, and fluorescence was determined at 510 nm (excitation) and 580 nm (emission). Alternatively, cells were loaded with dihydroethidine (5μM), a superoxide-specific fluorescent probe that, however, does not accumulate in mitochondria and thus reflects overall cellular superoxide levels.

Statistical evaluation. All measurements were performed in duplicate, and the mean value of these determinations from one animal was used as one data point. All data with isolated hepatocytes were obtained from three independent liver perfusions. Significant differences between means were determined by ANOVA/Tukey-Kramer Multiple Comparison posttest (InStat, GraphPad Software, San Diego, CA). The p values of ≤ 0.05 were considered significant.

RESULTS

Prolonged Treatment with Troglitazone Causes Liver Injury in Sod2$^{+/-}$ *Mice*

Normal healthy rats or mice can tolerate even excessively high oral doses of troglitazone (Herman *et al.*, 2002). Commensurate with these observations, treatment of wild-type (*Sod2*$^{+/+}$) mice with troglitazone (30 mg/kg/day, ip, for 4 weeks) was not associated with any apparent abnormalities in serum ALT activity (vehicle controls, 86 ± 28 U/l; troglitazone, 99 ± 34 U/l, $n = 5$–8 per group) or liver histopathology (Table 1).

In contrast, troglitazone was clearly hepatotoxic in *Sod2*$^{+/-}$ mice featuring underlying mitochondrial abnormalities. Preliminary dose finding studies had revealed that some mice died after > 4 weeks of continuous treatment with > 30 mg/kg. Therefore, groups of mice were administered troglitazone ip at a dose of 0, 10, or 30 mg/kg/day. At the end of the 4-week treatment period, the animals in the 30-mg/kg dose group exhibited mildly but significantly increased (twofold) serum ALT activity (Fig. 1A). Histopathological analysis of liver sections revealed that the mice exhibited liver damage to varying degree; some had developed large foci of midzonal hepatocellular necrosis (Fig. 2). The areas of necrosis were

TABLE 1
Hepatotoxicity Score in Wild-Type and *Sod2*$^{+/-}$ Mice Treated with Troglitazone for 4 Weeks

	Histopathology scores				
Treatment	0	1+	2+	3+	4+
Sod2$^{+/+}$					
Vehicle control ($n = 5$)	5	0	0	0	0
Troglitazone 30 mg/kg/day ($n = 5$)	5	0	0	0	0
Sod2$^{+/-}$					
Vehicle control ($n = 7$)	7	0	0	0	0
Troglitazone 10 mg/kg/day ($n = 7$)	3	4	0	0	0
Troglitazone 30 mg/kg/day ($n = 14$)	2	7	3	2	0

Note. Histological sections were obtained from livers of mice treated daily with ip injections of troglitazone in Solutol HS-15 (9%). Mice were sacrificed after 28 days. Slides stained with H&E were reviewed under low and high power by two individuals. Hepatocellular degeneration was graded as 0 (no degeneration); 1+ (small areas of degenerated hepatocytes); 2+ (moderate degeneration of midzonal hepatocytes); 3+ (severe degeneration of midzonal hepatocytes); and 4+ (panlobular confluent hepatocytic degeneration). Values are total number of animals featuring the indicated score.

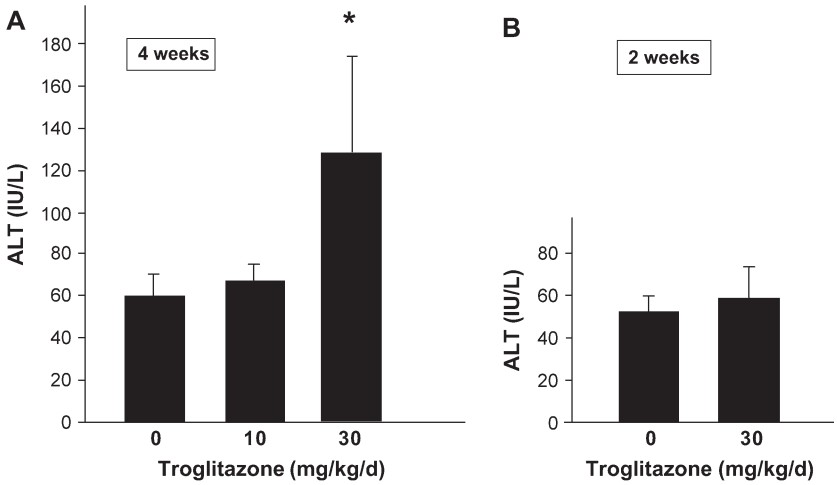

FIG. 1. Serum ALT activity of *Sod2*$^{+/-}$ mice treated daily with vehicle or troglitazone. (A) Troglitazone was administered for 4 weeks ($n = 7$–14 per group); (B) troglitazone was administered for 2 weeks ($n = 4$ per group). Data are means ± SD; *$p < 0.05$ versus vehicle controls.

congruent with TUNEL-positive hepatocytes (nuclei and cytoplasm), indicating the involvement of DNA cleavage (Fig. 3). The 10-mg/kg/day dose group featured only scattered small areas of necrotic hepatocytes, while the vehicle controls did not reveal any significant hepatic changes (Table 1).

To determine whether the hepatotoxic response triggered by troglitazone developed early and gradually or rather in a delayed manner, following a discreet lag period, a separate 2-week study was performed. *Sod2*$^{+/-}$ mice receiving 30 mg/kg/day troglitazone for 14 days did not exhibit any significant differences in serum ALT activity as compared to vehicle controls (Fig. 1B). Similarly, histopathological examination of liver sections did not reveal any changes (data not shown). These findings clearly indicate that the hepatotoxic response is not an acute one and that there is a lag phase of > 2 weeks after which liver injury becomes apparent.

Prolonged Treatment of Sod2$^{+/-}$ *Mice with Troglitazone Induces Oxidative Injury to Hepatic Mitochondria*

Because troglitazone has been shown to cause oxidant stress and mitochondrial alterations *in vitro* (Haskins *et al.*, 2001; Masubuchi *et al.*, 2006; Narayanan *et al.*, 2003; Shishido *et al.*, 2003; Tirmenstein *et al.*, 2002), we assessed the degree of mitochondrial injury in the liver of troglitazone-treated mice. One of the most sensitive markers of mitochondrial oxidative stress is aconitase, due to the enzyme's [Fe-S] clusters. Troglitazone dose dependently decreased aconitase activity (by 45% in the 30 mg/kg group) as compared to vehicle controls (Fig. 4A). Furthermore, we assessed the activity of complex I (NADH:ubiquinone oxidoreductase), another sensitive biomarker of mitochondrial oxidative stress (Williams *et al.*, 1998). Again, troglitazone significantly decreased complex I activity by 46% in both the low-dose and high-dose group, as compared to vehicle controls (Fig. 4B).

To provide further evidence of irreversible oxidant stress independent of [Fe-S] inactivation, we next determined mitochondrial protein carbonyl levels in the liver of troglitazone-treated mice. Increased carbonyls in amino acid residues are an established and sensitive marker of cumulative oxidative protein damage. We found that mitochondrial protein carbonyls were significantly increased in the 30 mg/kg group (+59%) (Fig. 4C). Taken together, these data indicate that troglitazone *in vivo* exerted a marked mitochondrial oxidant stress that critically impaired mitochondrial function.

Finally, we determined whether prolonged troglitazone exposure caused possible changes in GSH levels, the major antioxidant defense against oxidative stress and increased hydrogen peroxide formation in mitochondria (Fernandez-Checa and Kaplowitz, 2005). We found that the mitochondrial pool of GSH was significantly increased (+61% relative to vehicle controls) by treatment with 30 mg/kg troglitazone for 4 weeks (Fig. 5A), while the low dose had no apparent effect on GSH levels. Increased GSH steady-state levels are commonly occurring adaptive responses to sustained oxidant stress. This can be achieved by upregulation of GSH synthesis and/or by activation of uncoupling protein 2, which has been implicated in mitochondrial transport of GSH (Fernandez-Checa and Kaplowitz, 2005). Nevertheless, the higher GSH levels following troglitazone treatment were somewhat surprising, as one would expect that mitochondrial oxidant stress would initially decrease GSH. Therefore, we used an earlier time point and analyzed the mitochondrial GSH levels after 2-week exposure to troglitazone. Indeed, some individual mice in the high-dose group (30 mg/kg/day) exhibited decreased mitochondrial GSH levels, but the mean value did not significantly differ from that in vehicle control mice (Fig. 5B).

An important regulator of intramitochondrial oxidant stress is Sod2. Because mitochondrial Sod2 can potentially be induced by its substrate, superoxide anion, we ascertained whether the

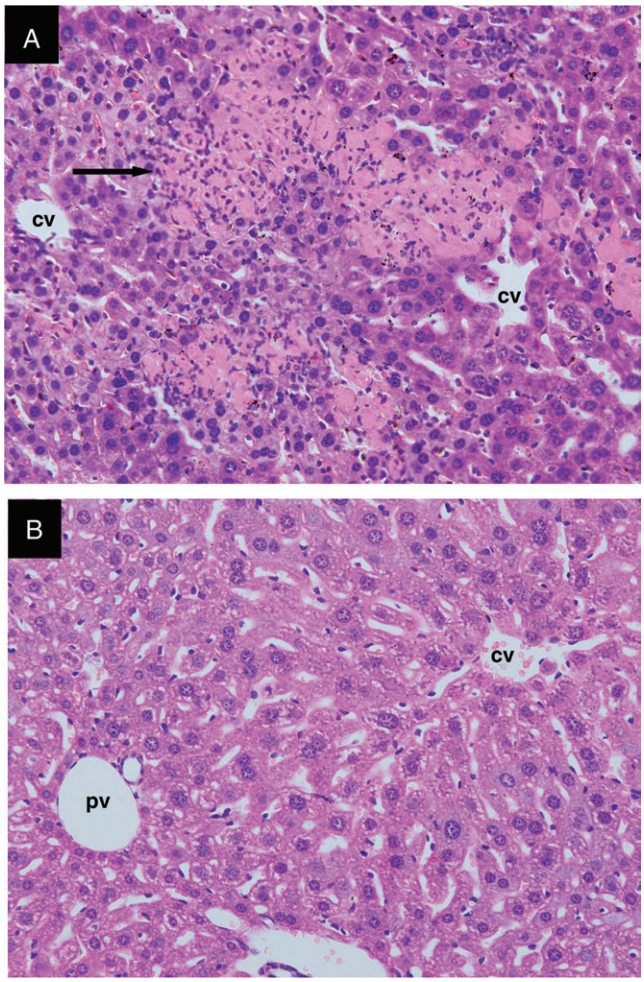

FIG. 2. Liver sections of *Sod2*$^{+/-}$ mice treated daily with troglitazone (30 mg/kg) (A) or vehicle (B) for 4 weeks. Note the areas of confluent hepatocellular necrosis in the 30 mg/kg group (arrow). H&E, original magnification ×40; cv, centrivenous; pv, perivenous.

oxidative changes that occurred in mitochondria after troglitazone administration had altered the translated Sod2 protein levels in the heterozygous mice. To this end, we compared the expression of Sod2 protein by immunoblotting in vehicle- and troglitazone-treated mice. Figure 6 shows that no treatment-related changes in the expression of Sod2 were apparent. This indicates that the ROS-dependent mechanisms of Sod2 induction were not activated by troglitazone and that the results were not confounded by differential expression of Sod2.

Troglitazone Produces Increased Mitochondrial Levels of Superoxide Anion in Hepatocytes Isolated from Sod2$^{+/-}$ *Mice*

The observed oxidative damage to mitochondria following repeated exposure to troglitazone could arise from oxidant stress directly generated in mitochondria or as a consequence of cytosolic oxidant stress generated from troglitazone metabolism, e.g., from thiol-reactive intermediates including the activated thiazolidinedione ring (He *et al.*, 2001; Kassahun *et al.*, 2001; Tettey *et al.*, 2001). Therefore, we explored whether troglitazone can directly increase mitochondrial net ROS production in mitochondria. To this end, we used MitoSOX Red, a superoxide anion-selective fluorescent probe that is targeted to mitochondria. We also compared the signals with those obtained with dihydroethidine, which allows for the detection of cytosolic sources of superoxide. Hepatocytes isolated from untreated wild-type (*Sod2*$^{+/+}$) and heterozygous (*Sod2*$^{+/-}$) mice were kept in short-term culture, exposed to various concentrations of troglitazone for 6 h (i.e., before any overt cell injury is apparent), and subsequently loaded with the fluorescent probes. In *Sod2*$^{+/-}$ mice, troglitazone markedly enhanced MitoSOX Red–derived fluorescence in a concentration-dependent manner, which became significant at 30μM and attained a two- to threefold increase over solvent controls at 100μM troglitazone (Fig. 7). In contrast, troglitazone (up to 100μM) did not increase MitoSOX Red fluorescence in hepatocytes from wild-type mice. Interestingly, troglitazone did not increase the cytosolic dihydroethidine signal at any concentration in either wild-type or mutant hepatocytes (data not shown). Collectively, these data indicate that troglitazone is associated with a mitochondria-selective increase in the net production of ROS that becomes manifest only in mice with functionally compromised mitochondria and that could explain the enhanced mitochondrial oxidative damage in *Sod2*$^{+/-}$ mice.

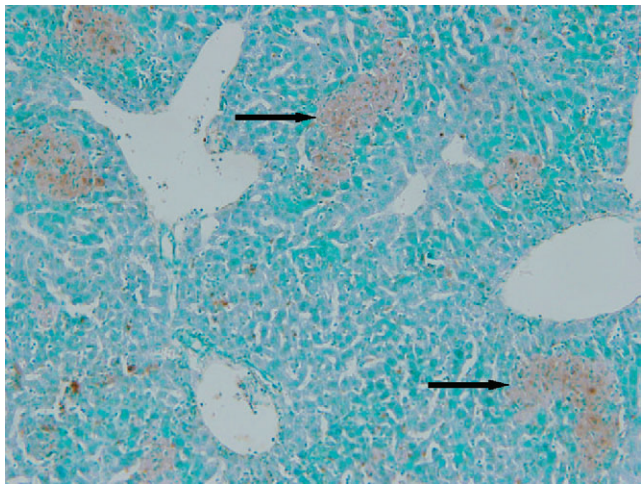

FIG. 3. TUNEL staining of liver sections of *Sod2*$^{+/-}$ mice treated daily with troglitazone for 4 weeks. Note the areas of TUNEL-positive cells which are congruent with the patches of hepatocellular necrosis (arrows). Original magnification ×40.

DISCUSSION

The major objective of this study was to utilize an underlying genetic mitochondrial alteration as a molecular tool to

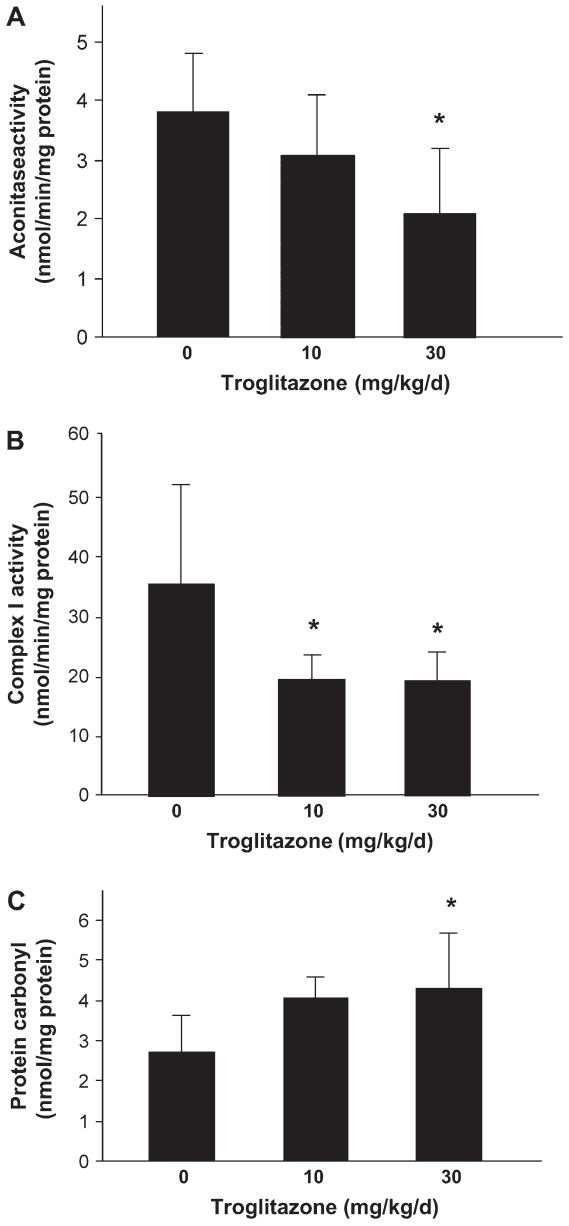

FIG. 4. Troglitazone-induced oxidative injury to hepatic mitochondria isolated from vehicle- or troglitazone-treated *Sod2*$^{+/-}$ mice. (A) Aconitase activity, (B) complex I activity, and (C) cumulative protein carbonyl content. Data are means ± SD ($n = 7$–14 per group); *$p < 0.05$ versus vehicle controls.

manipulate the mitochondrial redox state and to determine whether mice would be sensitized to the pro-oxidant and potential hepatotoxic effects of troglitazone. We found that prolonged administration of therapeutic doses of troglitazone to heterozygous *Sod2*$^{+/-}$ mice superimposed a distinct oxidant stress in liver mitochondria and that this was associated with delayed development of hepatic necrosis. Using this animal model, we show for the first time that troglitazone, which has caused idiosyncratic hepatotoxicity in patients, can induce hepatic necrosis if administered to mice with an underlying clinically silent genetic deficiency in mitochondrial function. Similar to the situation in susceptible patients, troglitazone hepatotoxicity was not an acute event (no apparent signs of hepatotoxicity after 2-week treatment) but instead was triggered after a lag time of several weeks. These findings are compatible with the concept of cumulative mitochondrial injury that ultimately translates into overt tissue damage after a critical point has been reached (threshold effect).

These conclusions were based on a number of observations. First, the end points used, including impaired aconitase and complex I activities and increased protein carbonyls, are all established biomarkers of enhanced mitochondrial oxidative stress. Second, liver injury did not occur in normal wild-type mice, but only in heterozygous mice whose antioxidant capacity in the mitochondrial matrix was compromised. Finally, *in vitro* exposure of troglitazone to hepatocytes from *Sod2*$^{+/-}$ mice resulted in acute net increases in superoxide anion as demonstrated with the mitochondia-selective fluorescent probe MitoSOX Red.

These data confirm and extend earlier studies demonstrating that a clear mitochondrial hazard is precipitated by troglitazone. For example, troglitazone has been known to decrease the $\Delta\Psi_m$, induce the mPT, and precipitate apoptosis in a number of cellular models (Haskins *et al.*, 2001; Masubuchi *et al.*, 2006; Tirmenstein *et al.*, 2002; Toyoda *et al.*, 2001; Yamamoto *et al.*, 2001). However, these studies were all conducted *in vitro*, using concentrations of troglitazone that highly exceed the therapeutic exposure levels.

The results of this *in vivo* mouse study highlight two aspects related to the liver liability of troglitazone. First, the data corroborate the concept that low doses of troglitazone are unable to produce hepatic mitochondrial changes in normal (wild-type) mice unless there is a clear genetic damage in the mitochondria's antioxidant defense system. This damage can be subtle yet severe enough to potentiate further mitochondrial injury superimposed by drugs that target the mitochondria and pose an additional stress. We have chosen heterozygous *Sod2* deficiency as a model because these mice gradually acquire oxidative damage to liver mitochondria due to sustained mitochondrial stress, but this condition remains unrecognized and the animals appear normal throughout their lifespan. Using this model, we sought to mimic a situation of underlying genetic abnormalities in mitochondria that might predispose individuals for increased vulnerability to drugs that are bioactivated in the liver and targeted to mitochondria.

The precise mechanisms that render *Sod2*$^{+/-}$ mice more sensitive to troglitazone than normal mice remain speculative. However, we and others have demonstrated that the mutant mice, due to a significant reduction in mitochondrial Sod2 expression, exhibit an enhanced basal level of mitochondrial superoxide (Ong *et al.*, 2006) as well as a decrease (by approximately 30%) of complex I activity (Liang and Patel, 2004; Melov *et al.*, 1999; Williams *et al.*, 1998). This is commensurate with the observations that *Sod2*$^{+/-}$ mice were

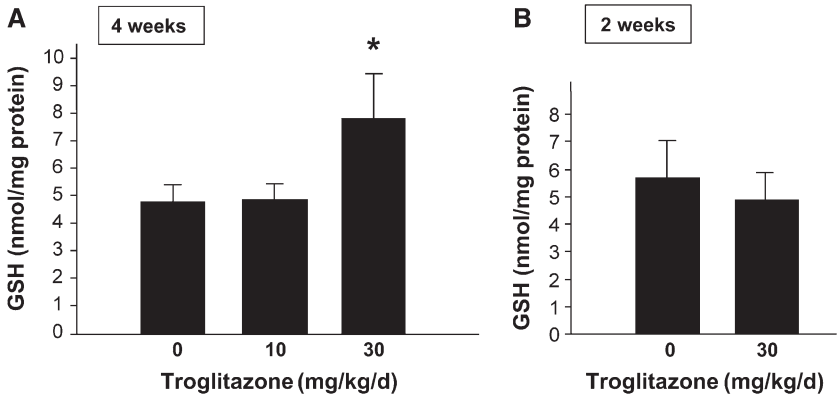

FIG. 5. GSH levels in mitochondria isolated from vehicle- or troglitazone-treated heterozygous *Sod2*$^{+/-}$ mice. (A) Troglitazone was administered for 4 weeks ($n = 7$–14 per group); (B) troglitazone was administered for 2 weeks ($n = 2$ weeks). Data are means ± SD; *$p < 0.05$ versus vehicle controls.

more sensitive to the pro-oxidant effects of nimesulide at low doses (Ong *et al.*, 2006). Thus, the underlying impairment of mitochondrial function most likely sensitizes hepatic mitochondria to an additional oxidant stress superimposed by a mitochondria-targeting drug such as troglitazone.

Exactly how troglitazone potentiates oxidant stress is not known. Here, we demonstrate for the first time that troglitazone produces superoxide selectively in the mitochondrial compartment. It is possible that net superoxide levels increased as a consequence of mPT opening and resulting loss of GSH, SOD1, and other antioxidants (Li *et al.*, 2006). Alternatively, ROS could be metabolism derived. For example, it has been shown that a major troglitazone metabolite in mice, rats, and humans is a quinone (Herman *et al.*, 2002), and quinones can potentially undergo redox cycling; however, this CYP3A4-dependent metabolite has not been related to any cytotoxic effects *in vitro* (He *et al.*, 2001; Tettey *et al.*, 2001). Furthermore, metabolism of troglitazone at the thiazolidinedione ring results in the formation of thiol-reactive electrophilic intermediates, yielding several glutathionyl-*S*-adducts (He *et al.*, 2004; Kassahun *et al.*, 2001; Prabhu *et al.*, 2002). This impairment of the pro-oxidant/antioxidant balance could also increase oxidant stress. Finally, an intriguing possibility is the concept of peroxidase-catalyzed formation of troglitazone phenoxy radicals. These radicals also consume GSH; their formation has, however, only been demonstrated in a cell-free model using HRP/H_2O_2 (Tafazoli *et al.*, 2005). Interestingly, the drug-related highly increased mitochondrial superoxide production became only detectable in hepatocytes from mice with compromised mitochondrial function, and not in normal

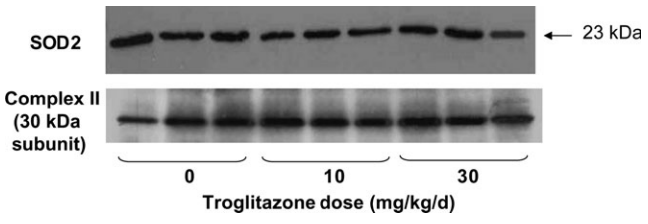

FIG. 6. Western immunoblot showing SOD2 expression in mitochondria isolated from *Sod2*$^{+/-}$ mice treated with vehicle or troglitazone for 4 weeks. Note the similar expression levels in all groups. Complex II/30 kDa subunit was used as loading control.

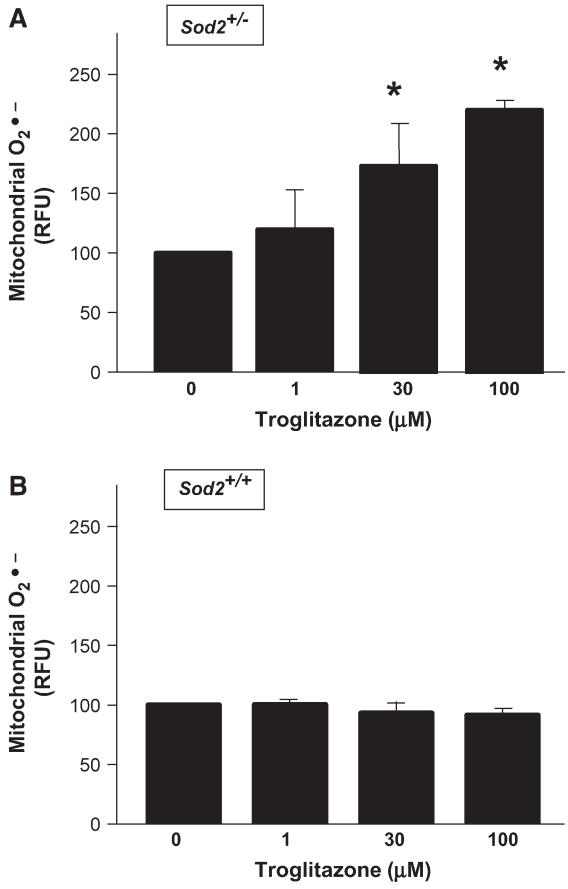

FIG. 7. Mitochondrial superoxide anion net production in cultured hepatocytes isolated from *Sod2*$^{+/-}$ (A) or *Sod2*$^{+/+}$ (B) mice. Hepatocytes were exposed to troglitazone or vehicle for 6 h, washed, and loaded with MitoSOX Red (5μM) for 10 min at 37°C. Fluorescence specific for mitochondrial superoxide anion was determined at 510/580 nm. Data are means ± SD of three independent liver perfusions; *$p < 0.05$ versus solvent controls.

mice. This could provide a mechanistic clue to the observation that only the *Sod2*$^{+/-}$ mice developed oxidative mitochondrial injury and hepatic necrosis.

The second aspect related to troglitazone-associated liver liability addresses a possible extrapolation of these murine studies to DILI in patients. Isolated experimental data that demonstrate a mitochondrial hazard *in vitro* cannot be easily reconciled with the clinical picture where the overwhelming majority of patients do not develop liver dysfunction. We have therefore speculated that an underlying genetic or acquired defect in mitochondria might predispose certain individuals to DILI because liver- and mitochondria-targeting drugs might exert a ''second hit,'' superimposed on a preexisting condition within the same subcellular compartment (Boelsterli, 2003). Interestingly, the sequelae of the inherited mitochondrial defects in *Sod2*$^{+/-}$ mice (the ''first hit'') increases with age (Kokoszka *et al.*, 2001; Van Remmen *et al.*, 2003), and age indeed seems to be a major apparent risk factor in humans, at least for certain drugs associated with idiosyncratic liver injury in patients.

Although polymorphic variants of SOD2 have been described in humans, the overall rationale for using this mouse model was not to mimic a specific human situation. Instead, we sought to utilize a generic model in which the downstream consequences of a number of inherited mitochondrial abnormalities would ultimately converge to compromised function of the complexes of the electron transport chain and induce cumulative oxidative damage to mitochondria, which would sensitize an individual to certain protoxicants.

In search of possible sensitizing factors (determinants of susceptibility) contributing to troglitazone-induced hepatic liability, a recent study has found a correlation between the incidence of liver injury and the combined GST-T1 and GST-M1 null genotype in Japanese patients with type 2 diabetes mellitus (Watanabe *et al.*, 2003). Although intriguing, this alone is unlikely to fully explain the increased risk for DILI among certain patient subsets. Mitochondrial abnormalities, which are not a rare event (Chinnery *et al.*, 2000; Shanske and Wong, 2004), could also play a role. In humans, the incidence of inherited mitochondrial disease is estimated to be approximately 1:5000, but presumably a much larger population is affected with somatic gene defects (e.g., mtDNA mutations) (Suzuki *et al.*, 1999).

In conclusion, the results of this study demonstrate that mice with a clinically inconspicuous genetic abnormality in mitochondrial function are sensitized to the pro-oxidant activity of repeated troglitazone administration which may unmask overt liver injury. It has been generally assumed that individual factors must play a role as determinants of susceptibility to DILI. With regards to mitochondria, it is therefore possible that underlying mitochondrial injury, including clinically silent genetic or acquired oxidative damage, may be such a risk factor that would lower the threshold for adverse effects of mitochondria-targeting drugs.

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