Peroxisome proliferator-activated receptor alpha (PPARα), a member of the nuclear receptor superfamily, controls the expression of a battery of genes involved in lipid homeostasis including those encoding peroxisomal and mitochondrial enzymes that carry out fatty acid catabolism. PPARα is mainly expressed in organs that are critical in fatty acid catabolism, such as liver, heart, and kidney.1-3 Perhaps the most critical role of PPARα is to modulate hepatic fatty acid catabolism. In untreated mice, PPARα controls constitutive expression of mitochondrial fatty acid β-oxidation enzymes.4 During periods of starvation in mice PPARα is activated, resulting in induction of both mitochondrial and peroxisomal fatty acid catabolism.5 Notably, in the course of spontaneous and ligand-induced activation of fatty acid catabolism excess H2O2 is produced as a byproduct of induction of peroxisomal acyl-CoA oxidase. Reactive oxygen species (ROS) are also produced during mitochondrial fatty acid β-oxidation. Although this increase in H2O2 is dealt with in part by catalase, glutathione peroxidase, and manganese superoxide dismutase, the cellular responses to ROS are saturated upon the massive

Abbreviations: ALT, alanine aminotransferase; APAP, acetaminophen; AST, aspartate aminotransferase; GSH, glutathione; NAPQI, N-acetyl-p-benzoquinone imine; PPARα, peroxisome proliferator-activated receptor alpha; ROS, reactive oxygen species; UCP2, uncoupling protein 2.

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activation of fatty acid catabolism that occurs after ligand activation of PPARx. Consequently, increased PPARx activity during accelerated fatty acid catabolism is associated with increased expression of free-radical scavengers such as catalase and Cu/Zn dismutase and mitochondrial uncoupling proteins (UCPs) that may serve to reduce mitochondrial ROS levels. Both direct and indirect effects suggest that PPARx may serve a protective role to combat the deleterious side effects of fatty acid catabolism, thus preserving, in particular, mitochondrial function.

Increased ROS levels are frequently associated with hepatotoxicity produced by overdose of drugs such as acetaminophen (APAP). APAP, the most common nonprescription analgesic used for pain relief and antipyresis, is a representative compound that causes liver toxicity upon overdose and is a significant public health concern due to occasional overdose in children and adults. A reactive quinone metabolite, N-acetyl-p-benzoquinone imine (NAPQI), generated by cytochrome P450-catalyzed oxidation, triggers hepatic toxicity by covalently binding with nucleophilic macromolecules and/or by elevating ROS leading to apoptosis and cell necrosis.

Interestingly, a connection between PPARx and APAP toxicity was established when it was discovered that pretreatment with clofibrate, a PPARx activator, protected mice against APAP-induced hepatotoxicity and that this protection was PPARx-dependent. Furthermore, it was recently reported that toxic doses of APAP inhibit fatty acid β-oxidation and that these effects were significantly reduced in mice lacking the major enzyme responsible for the bioactivation of APAP, CYP2E1, due, in part, to enhanced and persistent activation of PPARx and its target genes. Wildtype mice treated with APAP, however, showed suppressed PPARx activity. Thus, PPARx may function to protect mitochondria from ROS that occurs during APAP metabolism and as a natural consequence during fatty acid catabolism. In the present study the protective effects of PPARx activation during APAP-induced hepatotoxicity were further investigated and a role for the PPARx target gene UCP2 in mediating these protective effects explored.

Materials and Methods

Animals. Wildtype (C57Bl/6J) and ucp2-null (B6.129-Ucp2tm1Low/J) mice were obtained from the Jackson Laboratories (Bar Harbor, ME). Ppara-null mice and wildtype counterparts on the 129/Sv background were described previously. All animal experiments were carried out in accordance with the Institute of Laboratory Animal Resources guidelines and approved by the National Cancer Institute Animal Care and Use Committee.

Acetaminophen Experiments. Groups of 6 to 8-week-old male mice were fed W7y-14.643 (0.1%) diet for 24 hours before an intraperitoneal injection of APAP (400 mg/kg) dissolved in saline. All mice were euthanized by CO2 asphyxiation 2 hours, 6 hours, or 24 hours after the APAP dose. Livers were harvested and stored at −80°C before analysis. To assess liver damage, tissue was briefly washed with phosphate-buffered saline (PBS) and fixed in 10% neutral buffered formalin. Necrosis was scored by hematoxylin and eosin (H&E) staining. APAP-induced liver injury was determined by measuring aspartate aminotransferase (AST) and alanine aminotransferase (ALT) catalytic activities in serum using a commercial AST or ALT assay kit (Catalchem, Bridgeport, CT). Reduced glutathione (GSH) levels in liver were measured by a glutathione assay kit (Sigma-Aldrich, St. Louis, MO) and liver hydrogen peroxide (H2O2) levels were determined by use of the Peroxidetect kit (Sigma-Aldrich).

RNA Analysis. Dye-coupled complementary DNAs (cDNAs) were purified with a MiniElute PCR purification kit (Qiagen) and hybridized to an Agilent 44 K mouse 60-mer oligo microarray (Agilent Technologies, Santa Clara, CA). The data were processed and analyzed by Genespring GX software (Agilent Technologies). Significance was determined as >10-fold to the wildtype control samples. Specific messenger RNA (mRNA) levels were determined by quantitative real-time polymerase chain reaction (qPCR). RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and qPCR performed using cDNA generated from 1 μg of total RNA with SuperScriptII Reverse Transcriptase (Invitrogen). Primers for qPCR were designed using the Primer Express software (Applied Biosystems, Foster City, CA). qPCR reactions were carried out using the SYBR Green PCR master mix (SuperArray, Frederick, MD) and an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Quantitation was carried out using the comparative cycle threshold (CT) method and results were normalized to mouse β-actin.

Generation and Infection of the Adenovirus Constructs Expressing Recombinant UCP2. Rat UCP2 adenovirus was obtained from the Gene Transfer Vector Core (University of Iowa). For in vivo infection of recombinant adenoviruses, 6 to 8-week-old wildtype mice were intravenously injected in the
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Results

Wy-14,643 Pretreatment Protects Against APAP-induced Hepatotoxicity. Treatment of wildtype mice with APAP for 6 hours results in massive hepatic toxicity as revealed by gross morphology of the liver (Fig. 1A), increased ALT and AST enzyme levels (Fig. 1B), and faint pericentral and periportal H&E staining of liver parenchyma (Fig. 1C). Pretreatment with Wy-14,643 for 24 hours before APAP treatment results in total protection against APAP toxicity; Wy-14,643 treated mice had no evidence of liver damage. At 24 hours post-APAP treatment, Wy-14,643-treated mice were still protected, as indicated by reduced ALT enzyme levels and normal liver histology (Supporting Fig. 1). In contrast, Ppara-null mice exhibited no Wy-14,643 protection against APAP toxicity (shown by increased ALT and AST activities), indicating that the protection was PPARα-dependent (Fig. 2A). To demonstrate that the effect was not specific to the experimental ligand Wy-14,643 and to mouse PPARα, PPARα-humanized mice (a human PPARα gene introduced in the Ppara-null background) treated with

Statistical Analysis. Statistical analysis was performed using GraphPad Prism (San Diego, CA). Analysis of variance (ANOVA) with Bonferroni’s multiple comparison test was used to compare the various groups. P < 0.05 was considered significant.

Quantitation of APAP Metabolites and Serum Palmitoylcarnitine. APAP serum metabolites (APAP, APAP-NAC, APAP-glucuronide, APAP-CYS) were monitored as described. For serum palmitoylcarnitine, deproteinated serum samples from wildtype mice were analyzed by use of an API2000 triplequadrupole mass spectrometer (Applied Biosystems). Debrisoquine was used as internal standard. Samples were injected into a high-performance liquid chromatography (HPLC) system (PerkinElmer, Waltham, MA) using a Luna C18 column (Phenomenex, Torrance, CA; 50 x 2.1 mm i.d.). The flow rate through the column at ambient temperature was 0.3 mL/min with a gradient (methanol:water:acetonitrile, containing 0.1% formic acid) from 5:60:35 to 5:5:90 in a 6-minute run. The column was equilibrated for 1.5 minutes before each injection. The mass spectrometer was operated in the turbo ion spray mode with positive ion detection; the turbo ion spray temperature was maintained at 350°C and a voltage of 4.5 kV was applied to the sprayer needle. Nitrogen was used as the turbo ion spray and nebulizing gas. Detection and quantification were performed using the multiple reactions monitoring mode, with m/z 400.3/85.0 for palmitoylcarnitine and m/z 176.1/134.2 for debrisoquine. Using Analyst software (Applied Biosystems), serum palmitoylcarnitine concentrations were determined by calculating the ratio between the peak area of palmitoylcarnitine and the peak area of debrisoquine and fitting with a calibration curve with a linear range from 10 nM to 1 μM (r = 0.99).

Western Blot Analysis. Liver whole cell or mitochondrial extracts were prepared and subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Membranes were incubated with antibodies against CYP2E1, total JNK, p-JNK (Cell Signaling Technologies, Danvers, MA), or UCP2 (Santa Cruz Biotechnology, Santa Cruz, CA). JNK kinase assays were performed using the nonradioactive SAPK/JNK kinase assay kit (Cell Signaling Technologies) according to the manufacturer’s instructions.
fenofibrate were also protected (Fig. 2B). However, mice treated with the anti-Fas antibody Jo-2 to stimulate Fas receptor-mediated apoptosis were not protected from Wy-14,643 pretreatment (Supporting Fig. 2). Pretreatment with Wy-14,643 did not significantly impact APAP metabolism, as demonstrated by serum profiling of APAP and its metabolites (APAP-NAC, APAP-glucuronide, APAP-CYS) 2 hours after APAP administration (Supporting Fig. 3).

**Wy-14,643 Blocks APAP-induced Hepatic Stress Response Genes.** In order to understand the transcriptional responses associated with toxic doses of APAP-treatment and potential targets whereby PPARα was mediating its protective affects, microarray analysis was carried out on liver mRNA from 6 hours APAP-treated and Wy-14,643-pretreated/APAP-treated Ppara-null mice. A total of 53 genes were up-regulated by APAP and 45 genes up-regulated by Wy-14,643/APAP; 14 genes were up-regulated by both treatments (greater than 10-fold). Most interesting was the marked induction and suppression of c-fos and c-jun expression upon APAP treatment and Wy-14,643-pretreatment prior to APAP administration, respectively (Fig. 3A). qPCR analysis confirmed that c-fos and c-jun mRNAs were robustly induced by APAP and suppressed by Wy-14,643-pretreatment prior to APAP (Fig. 3B). Wy-14,643-pretreatment also blocked APAP-induced phosphorylation of JNK, an important signaling component of APAP-induced toxicity.20-22 Wildtype mice treated with APAP for 6 hours exhibited increased p-JNK that was not found with Wy-14,643-pretreatment, whereas Ppara-null mice have increased p-JNK following Wy-14,643-pretreatment (Fig. 3C). To ensure that p-JNK was associated with increased activity, kinase assays were performed and increased p-JNK levels were indeed consistent with elevated p-c-jun levels (Fig. 3C, bottom panel).

**Wy-14,643 Blocks APAP-Induced Oxidative Stress.** APAP treatment results in a decrease in hepatic levels of GSH at 2 hours and 6 hours, due in part to the production of the quinone NAPQI from APAP that is rapidly neutralized by GSH conjugation by glutathione S-transferase. This decrease was partially restored by Wy-14,643-pretreatment. However, the maintenance of GSH levels was even more pronounced in isolated mitochondria (Fig. 4A). H2O2 levels are inversely correlated with GSH levels and reflect increase oxidative stress. Indeed, Wy-14,643-pretreatment decreased H2O2 levels elevated by APAP treatment, and this was most pronounced in isolated mitochondria (Fig. 4B).

APAP toxicity is also associated with increased levels of long-chain acylcarnitines in serum that are likely due to mitochondrial damage.15 Metabolomics comparison of serum revealed marked differences in serum metabolites between APAP-treated and Wy-14,643-pretreatment/APAP as indicated by the scores plot separation of the two groups (Supporting Fig. 4A). This difference was driven, among others, by differences in levels of palmitoylcarnitine that were elevated in APAP-treated mouse serum and normal in Wy-14,643-pretreatment/APAP (Supporting Fig. 4B). Pretreatment with Wy-14,643 prior to APAP administration blocks the increase in palmitoylcarnitines, as indicated by direct quantification of palmitoylcarnitine (Fig. 4C).

At 2 hours post-APAP treatment, both APAP- and Wy-14,643/APAP-treated mice exhibited extensive GSH depletion in both the liver and mitochondria (Fig. 4D).

**Role of UCP2 in Wy-14-643 Protection Against APAP-Induced Hepatotoxicity.** Because APAP toxicity results in elevated mitochondrial oxidative stress and mitochondrial damage, a role for UCP2 in Wy-14,643...
protection against APAP-induced hepatic damage was investigated. UCPs are located in the mitochondrial inner membrane and are associated with decreased hepatic ROS. Wy-14,643 treatment induced UCP2 mRNA in the absence and presence of APAP (Fig. 5A, left panel); similar induction was not observed in Ppara-null mice. Protein levels of UCP2 were also measured in mitochondrial extracts from control and mice treated with Wy-14,643 for 24 hours (Supporting Fig. 5). To determine whether UCP2 has a role in Wy-14,643 protection against APAP hepatotoxicity, Ucp2-null mice were subjected to Wy-14,643 and APAP treatment. Mice lacking expression of UCP2 were not protected against APAP hepatotoxicity following Wy-14,643, as revealed by serum ALT and AST enzyme levels (Fig. 5B) and liver histology (Fig. 5C). The Ucp2-null mice were still responsive to Wy-14,643, as indicated by induction of the PPARx target genes Mcad, Cpt1, and Pdk4 (Fig. 6A), indicating that the loss of the protective effects of Wy-14,643 in this model is due to the lack of UCP2 and not PPARx itself or other PPARx target genes. Wy-14,643 administration to Ucp2-null mice also did not restore mitochondrial GSH loss upon APAP treatment (Fig. 6B) nor fully suppressed the increase in p-JNK (Fig. 6C). These results suggest that the PPARx target gene UCP2 may be responsible for the protective effect of PPARx activators against APAP-induced toxicity.

Ucp2-null mice could have subtle changes that render them resistant to the Wy-14,643 protection that are unrelated to UCP2. In order to further establish a role for UCP2 in Wy-14,643-induced protection against APAP hepatotoxicity, forced expression of the protein in livers of wildtype mice was carried out. Recombinant adenoviruses expressing UCP2 (Ad-Ucp2) were constructed and infused into the mouse
livers prior to administration of APAP; UCP2 protein was robustly expressed in the livers of wildtype mice (Fig. 7, bottom right panel). Mice receiving the Ad-\textit{Ucp2} were protected against APAP-induced liver toxicity as revealed by H&E staining showing protection against liver necrosis (Fig. 7), lower serum AST and ALT enzyme activities (Fig. 8A), increased mitochondrial GSH (Fig. 8B), and lower p-JNK levels (Fig. 8C). This protection by Ad-\textit{Ucp2} was evident at 24 hours post-APAP treatment as indicated by reduced levels of ALT enzyme (Fig. 8D). No protection was found when the control adenovirus expressing Cre recombinase was used and adenovirus itself did not appear to influence CYP2E1 expression (Supporting Fig. 6). ALT activity values for Ad-\textit{Cre}/APAP-treated mice ranged from 1.8 to 8.0 U/mL, whereas values from Ad-\textit{Ucp2}/APAP-treated mice ranged from 0.06 to 0.7 U/mL. Even the highest Ad-\textit{Ucp2}/APAP ALT activity value was still 2.6 times lower than the lowest Ad-\textit{Cre}/APAP value. These data suggest that UCP2 can protect against APAP-induced hepatotoxicity and that it is a critical target gene responsible for PPAR\textit{\alpha}-mediated protection during APAP-induced hepatotoxicity.

\textbf{Discussion}

The present study demonstrates a novel, protective role for PPAR\textit{\alpha} during APAP-induced hepatotoxicity and sheds mechanistic insight into the importance of UCP2 in mediating these protective effects. When the experimental agonist Wy-14,643 was administered prior to a toxic dose of APAP, wildtype mice were completely protected against hepatotoxicity, as revealed by gross liver morphology, H&E-stained liver sections showing no significant liver damage, and low serum AST and ALT enzyme levels. In addition, livers from mice pretreated with Wy-14,643 prior to APAP had decreased oxidative stress, as revealed by lower \textit{H}_{2}\textit{O}_{2} levels and higher GSH levels compared with livers from mice only treated with a toxic dose of APAP at 6
hours. Interestingly, Wy-14,643-treated mice exhibited a rapid reduction of GSH levels at 2 hours post-APAP treatment. In fact, these results are in strong agreement with a previous report demonstrating that mice lacking CYP2E1 were protected from APAP-induced hepatotoxicity due in part to activation of PPARα. Evidence for decreased oxidative stress was also revealed by reduced induction of c-jun and c-fos and lower p-JNK levels upon Wy-14,643 pretreatment. This effect was specific to APAP-induced hepatotoxicity and JNK pathway attenuation, as Jo-2 treatment that stimulates the Fas death pathway was unaffected by pretreatment with Wy-14,643. This observation is consistent with a previous report demonstrating that attenuating JNK signaling did not protect from Fas-mediated cell death. In general, it is the enhanced...
and persistent PPARα activation prior to APAP treatment that is important for mediating these effects. However, studies examining the role of PPARα activation post-APAP treatment should be conducted to determine if this pathway holds any promises for therapeutic intervention.

Previous studies revealed that acylcarnitines were elevated early after APAP treatment and that their elevation was indicative of mitochondrial damage and dysfunction.\(^{15,19}\) In the present study these observations were confirmed, as palmitoylcarnitine was elevated by toxic doses of APAP and maintained at normal levels (compared with untreated controls) by Wy-14,643 pretreatment. The enhanced toxicity in the Ppara-null mice revealed that the protective response to Wy-14,643 was PPARα-dependent. Protection of APAP toxicity by Wy-14,643 also extended to human PPARα, as indicated by similar protection from APAP-induced hepatotoxicity in PPARα-humanized mice receiving the PPARα activator fenofibrate. PPARα activates a large number of target genes primarily associated with fatty acid transport and catabolism. Thus, it was important to determine which among these target genes afforded protection. Earlier studies revealed that among the earliest events associated with APAP toxicity was elevated oxidative stress as a result of oxidation of APAP to the quinone metabolite NAPQI by cytochromes P450, notably by
UCP2 facilitates this protection are warranted and will provide greater understanding by which ROS elevating hepatocants, such as APAP, mediate their effects.

Another intriguing possibility was demonstrated in a recent study where the authors reported an intimate relationship between elevated ROS and UCPs. UCP2 (and UCP3) contain reactive cysteines that can be modified by GSH. The deglutathionylation/glutathionylation regulates UCP2 and UCP3 activity. In the presence of elevated ROS, GSH is depleted and the proteins lose the conjugated glutathione, thereby rendering them active and able to neutralized ROS. Under the conditions of APAP-induced hepatotoxicity, elevated ROS levels likely mediate similar activation of UCP2, however, only following activation by PPARα.

In conclusion, this study adds to our understanding of how toxic doses of APAP mediate hepatotoxicity and provides new insight into the importance of PPARα activation in maintaining proper mitochondrial function, most likely through UCP2 under normal and pathologic conditions. Further, this study lends even greater support for how repression of PPARα activation can lead to deleterious effects. Using Ucp2-null mice and mice transiently expressing UCP2 (from adenovirus), a convincing role for UPC2 in protecting against APAP-induced hepatotoxicity through preservation of mitochondrial function was demonstrated. Further studies to determine the mechanisms by which UCP2 facilitates this protection are warranted and will provide greater understanding by which ROS elevating hepatocants, such as APAP, mediate their effects.
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