3,5,5-Trimethyl-Hexanoyl-Ferrocene Diet Protects Mice from Moderate Transient Acetaminophen-Induced Hepatotoxicity

Mi Sun Moon,* Boo-Hyon Kang,|| Jacek Krzeminski,† Shantu Amin,† Cesar Aliaga,‡ Junjia Zhu,§ Emily I. McDevitt,* Susan Kocher,* John P. Richie,§ and Harriet C. Isom*¶

*Department of Microbiology and Immunology, †Department of Pharmacology, ‡Department of Biochemistry and Molecular Biology, §Department of Public Health Sciences, ¶Department of Pathology, Milton S. Hershey Medical Center, The Penn State College of Medicine, Hershey, Pennsylvania 17033; ||Preclinical Research Center, Chemon Inc., Jeil-Ri, Yangji-Myeon, Cheoin-Gu, Yongin-Si, Gyeonggi-Do, Korea

1 Corresponding author:
Penn State Cancer Institute, CH72
Department of Microbiology and Immunology
Penn State College of Medicine
Penn State Milton S. Hershey Medical Center
P.O. Box 850
500 University Drive
Hershey, PA 17033-0850

Telephone: (717) 531-5877
Fax: (717) 531-5103
E-mail: hisom@psu.edu

© The Author 2011. Published by Oxford University Press on behalf of the Society of Toxicology.
All rights reserved. For Permissions, please email: journals.permissions@oup.com
ABSTRACT

Acetaminophen (APAP) overdose is the most frequent cause of adult acute liver failure. Susceptibility or resistance to APAP toxicity is most likely accounted for by the interplay of several factors. One factor important in multiple different chronic liver diseases that may play a role in APAP toxicity is elevated hepatic iron. Hereditary hemochromatosis is traditionally associated with hepatic iron overload. However, varying degrees of elevated hepatic iron stores observed in chronic hepatitis C and B, alcoholic liver disease and nonalcoholic fatty liver disease also have clinical relevance. We employed an animal model in which mice are fed a 3,5,5-trimethyl-hexanoyl-ferrocene (TMHF)-supplemented diet to evaluate the effect of elevated hepatic iron on APAP hepatotoxicity. 300 mg/kg APAP was chosen because this dosage induces hepatotoxicity but is not lethal. Since both excess iron and APAP induce oxidative stress and mitochondrial dysfunction, we hypothesized that the TMHF diet would enhance APAP hepatotoxicity. The results were the opposite. Centrilobular vacuolation/necrosis, APAP-adducts, nitrotyrosine adducts, and a spike in serum alanine aminotransferase, which were observed in control mice treated with APAP, were not observed in TMHF-fed mice treated with APAP. Further analysis showed that the levels of CYP2E1 and CYP1A2 were not significantly different in TMHF-treated compared to control mice. However, the magnitude of depletion of glutathione following APAP treatment was considerably less in TMHF-treated mice than in mice fed a control diet. We conclude that a TMHF diet protects mice from moderate transient APAP-induced hepatotoxicity prior to the formation of APAP adducts and one contributing mechanism is reduction in glutathione depletion.

KEY WORDS: hepatic iron overload; acetaminophen; hepatotoxicity; glutathione; CYP2E1;
INTRODUCTION
Acetaminophen (N-acetyl-p-aminophenol, APAP) is a commonly used analgesic/antipyretic which is safe at therapeutic doses; however, in overdose, APAP produces a potentially fatal fulminating centrilobular hepatic necrosis, which can bridge to the periportal regions of the liver lobule. In the United States alone, acetaminophen toxicity results annually in more than 50,000 emergency room visits, several thousand hospitalizations and between 400 and 500 deaths. In the late 1990’s, the adult Acute Liver Failure (ALF) Study Group initiated a study to determine the etiology of ALF in adults. Between January 1998 and July 2007, 1,147 patients at 23 clinical sites in the United States were enrolled. Acetaminophen overdose accounted for 46% of the cases, thereby representing the most frequent cause of adult ALF.

It has been estimated that about half of acetaminophen ALF cases are caused by accidental or unintentional ingestion of multiple doses (that are each less than a suicidal dose) over several days. When 275 cases of ALF due to acetaminophen overdose were reviewed, 122 were intentional, 131 unintentional and 22 could not be categorized. Whether the overdose was intentional or unintentional, the mortality without transplantation was the same, 27%. The cases where the acetaminophen dose does not exceed the amount recommended on the packaging label probably represents 10% of those reaching ALF, although the numbers are uncertain. Indeed, data from a placebo controlled clinical study showed that 38% of healthy adult volunteers who were administered the maximum therapeutic dose of acetaminophen (4 g/d for 14 days), in an inpatient setting, exhibited transient asymptomatic elevations in serum alanine aminotransferase (ALT) levels that were three times the upper limit of normal. None of the participants given a placebo had a maximum of ALT of more than three times the upper limit of normal. A more recent study with a cohort of healthy adults that received APAP at 4 g/d for 7 days showed that one third of the individuals had transient ALT levels exceeding two fold baseline indicative of
liver injury, confirming the previous report.

The reasons for unintentional overdose are complex. A major cause is lack of awareness of overdose because of the presence of APAP in products other than Tylenol, in particular APAP/narcotic combinations and APAP in cold medications. Indeed, APAP is found in more than 300 products. However, it is also important to note that individuals have been described that consume massive daily quantities of APAP without hepatic injury. Susceptibility or resistance to toxicity by a specific dose of APAP is most likely accounted for by the interplay of multiple genetic and environmental factors. These include race, gene polymorphisms, alterations in immunity, viral infections, alcohol abuse, obesity, age, drug interactions and chronic liver diseases. Indeed, any factor that alters liver gene expression directly or indirectly involved in APAP detoxification/metabolism could increase or decrease hepatic sensitivity to APAP. One factor, present in multiple different chronic liver diseases, which may play a role in susceptibility or resistance to APAP toxicity, is elevated hepatic iron. Excess iron alters liver gene expression in many ways including inducing oxidative stress, causing mitochondrial dysfunction, and increasing hepatocyte proliferation.

Iron overload has been associated with numerous liver diseases. The genetic disease hereditary hemochromatosis (HH) traditionally associated with hepatic iron overload resulting in liver injury and increased incidence of hepatocellular carcinoma (HCC) has been well studied. However, varying degrees of elevated hepatic iron stores or “iron overload” observed in other liver diseases also have clinical relevance. These include chronic hepatitis C, chronic hepatitis B, alcoholic liver disease and nonalcoholic fatty liver disease. A recent retrospective study of nonalcoholic steatohepatitis patients with and without HCC showed that iron deposition in the liver was more frequent in patients with HCC than in HCC free controls. The subjects in the
study did not have HH and the iron excess was mild to moderate.

It is clear that elevated hepatic iron levels play a role in multiple different chronic liver diseases. Elevated hepatic iron alters liver gene expression and as such, could increase or decrease hepatic sensitivity to APAP. We hypothesized that elevated hepatic iron would increase hepatic sensitivity to APAP and employed an animal model in which mice are fed a 3,5,5-trimethyl-hexanoyl-ferrocene (TMHF)-supplemented diet to test this hypothesis. The results clearly demonstrate the contrary, specifically, that feeding mice a TMHF-supplemented diet protected against APAP toxicity.
MATERIALS AND METHODS

Animals. Eight week-old male C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). The animals were acclimated for approximately 1 week prior to the experiments. All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Institutes of Health standards. At 12 weeks of age, mice were fed with either control diet or 3,5,5-trimethyl-hexanoyl-ferrocene (TMHF) diet for 4 weeks. The control diet was modified from AIN-93M diet (ICN, Irvine, CA), while the TMHF diet was achieved by supplementing control diet with 0.5% TMHF (Organic Synthesis Core, Penn State Hershey Cancer Institute, Hershey, PA). Animals were fasted overnight (16-18 h) and then received an intraperitoneal injection of warm saline (control mice) or warm saline containing 300 mg/kg APAP (Sigma, St. Louis, MO).

Animal protocol. At various time points post APAP injection, animals were sacrificed and blood was collected by cardiac puncture. Blood samples then were allowed to clot overnight at 4°C and centrifuged to obtain serum for ALT activity. Immediately after collecting blood, the liver was excised and weighed. A small portion of liver from each mouse was fixed in 10% phosphate buffered formalin and a portion of the remaining liver was snap-frozen and stored at -80°C for Western blot analysis.

Histology. Formalin fixed liver tissue sections were paraffin embedded and subsequently mounted onto glass slides for hematoxylin and eosin (H&E) staining as well as immunohistochemical analysis. H&E stained liver sections were examined by a pathologist using light microscopy. Centrilobular vacuolation/necrosis induced by APAP treatment was
scored according to the following. The grading refers to the area of lesion/area of liver lobe observed: grade 0 (normal); grade 1 (minimal; area of lesion, 0 to ≤ 1/4); grade 2 (slight; area of lesion, 1/4 to ≤ 1/2); grade 3 (moderate; area of lesion, 1/2 to ≤ 3/4); grade 4 (severe; 3/4 to ≤ 1) at x 40 magnification. Iron pigmentation was scored according to a grading scheme: grade 0 (normal); grade 1 (minimal, 1~10 cells); grade 2 (slight, 11~20 cells); grade 3 (moderate, 21~30 cells); grade 4 (severe, ≥ 30 cells) at x 400 magnification.

**ALT assays.** Liver toxicity was quantified by measuring ALT levels in the serum using a diagnostic kit (Biotron Diagnostics, Inc., Hemet, CA). Briefly, pre-warmed ALT substrate (500 µL) was added to 10 µL of serum from each sample and incubated in a water bath at 37°C for 30 min. Color Reagent A (500 µL) was added to each sample and incubated in a water bath at 37°C for 10 min. Color Developer B (2 mL) was subsequently added to each sample, and was reincubated in a water bath at 37°C for 5 min. The absorbance of the samples was measured by spectrophotometer at a wavelength of 505 nm. The ALT levels were then calculated according to the manufacturer’s instructions.

**Glutathione (GSH) detection.** Total GSH in the liver was determined using a GSH assay kit (Oxford Biomedical Research Inc., Rochester Hills, MI). Briefly, liver tissues were rinsed in ice-cold phosphate buffered saline (PBS) and homogenized in 500 µl assay buffer. After the homogenate was centrifuged at 10,000 x g for 15 min, supernatants (50 µl) were transferred into 350 µl ice-cold 5% metaphosphoric acid. Precipitated proteins were removed by centrifugation at 1,000 x g for 10 min, and the supernatant was diluted with assay buffer prior to analysis. 50 µl
of diluted supernatant was mixed with DTNB (50 µl) and reductase solution (50 µl) in a 96 well plate and incubated for 5 min. After adding 50 µl of NADPH, the absorbance was measured at 412 nm for 10 min with 1 min intervals by a kinetic microplate reader (Synergy HT, BioTek Instruments, Inc., Winooski, UT). The total GSH concentration was then calculated according to the manufacturer’s instructions.

**Perls’ Prussian blue staining.** Formalin-fixed paraffin-embedded tissue samples were incubated twice in xylenes (Sigma) (5 min each) and then rehydrated by incubating twice (5 min each) in graded alcohols (100%, 95%, 70% ethanol) before incubating twice (5 min) in dH₂O. Rehydrated tissue was incubated in 10% potassium ferrocyanide (Sigma): 20% HCl (1:1) for 15 min and then rinsed with dH₂O. Tissues were then counterstained in nuclear fast red (Sigma; 0.5% nuclear fast red in 5% aluminum sulfate) for 5 min. Tissues were rinsed once in dH₂O and then in 100% ethanol. The slides were photographed by light microscopy.

**Microsome isolation.** Frozen liver tissue was homogenized in microsome preparation buffer containing 150 mM KCl, 10 mM EDTA, and 20 mM Tris (pH7.5). The homogenates were then centrifuged at 10,000 x g for 20 min at 4°C. The supernatants were transferred to polyallomer tubes (Beckman Coulter Inc., Brea, CA) and centrifuged at 104,000 x g for 60 min at 4°C. The pellets were then washed with microsome preparation buffer and resuspended in microsome storage buffer containing 50 mM Tris (pH7.5), 1 mM EDTA, 20% glycerol, and 1 mM DTT. Microsomal fractions were stored at -80°C until use.
**Western blot analysis.** Liver tissue was homogenized in Radio-Immunoprecipitation Assay buffer containing 100 μL/mL Protease Inhibitor Cocktail (Sigma), 0.02 mM phenylmethanesulfonyl fluoride, and 50 μL/mL Phosphate Inhibitor Cocktail I and II (Sigma). The homogenates were then centrifuged at 6,400 x g for 10 min at 4°C. The bicinchoninic acid assay was used to determine protein concentrations. 20 μg of protein from each sample was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and separated proteins were then transferred to a nitrocellulose membrane. The membranes were incubated in a blocking solution containing 5% milk in tris-buffered saline-tween for 1 h, followed by incubation with primary antibody against ferritin (Abcam, Cambridge, MA) APAP (Abcam), Nitrotyrosine (Abcam), or β-actin (Cell Signaling Technology, Beverly, MA) overnight at 4°C. 10 μg of microsomal protein from each sample was analyzed as described above and incubated with primary antibody against cytochrome P450 (CYP) 2E1 (Abcam), CYP1A2 (Abcam), or β-actin (Cell signaling Technology). The membranes were then washed and incubated with a horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Bound proteins were visualized by enhanced chemiluminescence (Perkin Elmer Inc., Waltham, MA). The intensity of bands was quantified by densitometry.

**Immunohistochemical detection.** Formalin-fixed, paraffin-embedded tissue sections were baked at 60°C for 1 h and deparaffinized by passing through xylene, followed by washing in a series of graded alcohols. Slides were boiled in antigen unmasking solution (Vector Laboratories, Burlingame, CA) for 10 min, followed by incubation in 3% hydrogen peroxide (Fisher Scientific, Pittsburgh, PA) for 10 min to inactivate endogenous peroxidases. Sections were blocked in 5% BSA in PBS-T and then incubated overnight at 4°C with primary anti-APAP (Abcam), or
nitrotyrosine antibody (Abcam). Sections were washed in PBS and subsequently incubated in biotinylated universal antibody for 1 h. Antibody binding was visualized by the Universal Elite ABC kit (Vector Laboratories), using 3,3’-Diaminobenzidine (DAB) as a substrate (Vector Laboratories) according to the manufacturer's instructions. Hematoxylin solution (Vector Laboratories) was used for counterstain. As a negative control, the sections were incubated in secondary antibody only.

**Statistical analysis.** ANOVA model with post-hoc Dunnett’s tests were performed for the comparison of each time level to the initial level (0 h) within group. The control and TMHF groups within the same time level were compared using analysis of simple effects inside the ANOVA model. Data were displayed as mean ± SD (or SE). A p-value of < 0.05 was defined as statistically significant. All statistical analyses were conducted with SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

**RESULTS**

*Mice Treated with TMHF Demonstrate Hepatic Iron Overload*

Mice treated with either control or TMHF diet for 4 wks were injected with 300 mg/kg APAP and sacrificed at 0, 3, 6, 12, 24 h post APAP treatment. To determine the degree and distribution of hepatic iron deposition, liver tissues from either control or TMHF-treated mice treated or not treated with APAP were examined by Perls’ Prussian blue staining (Fig. 1A). Tissue sections from control mice did not display positive staining for iron deposits; however, liver sections from TMHF-treated mice displayed strong positive staining for iron deposits throughout the liver tissue section at all time points post APAP injection. Positive staining was
exhibited primarily in hepatocytes, as well as some non-parenchymal cells, indicating that TMHF successfully induced iron overload in this mouse model. Moreover, all H&E stained liver tissue sections from TMHF-fed mice with or without APAP treatment exhibited spots of brown pigment, indicating iron deposits, while stained liver sections from control mice did not (Table 1).

Western blot analysis was performed to determine ferritin levels, an iron storage protein. It is well known that ferritin is regulated by intracellular iron concentration at the post-transcriptional level. As expected, the level of ferritin protein was dramatically elevated in liver from mice-treated with TMHF diet compared to mice fed a control diet regardless of APAP treatment (Fig. 1B). These data indicate that TMHF containing diet successfully induced iron overload in mice, and is accompanied by the appropriate physiologic changes in ferritin levels.

**Mice Fed a TMHF-Supplemented Diet Demonstrate Hepatomegaly**

To determine whether TMHF treatment alters tissue mass, whole livers were excised and weighed at time of sacrifice. The relative liver weight from all mice fed a control diet were approximately 3 to 4% of the body weight, while all TMHF-fed mice showed a dramatic increase (6 to 7%) in relative liver weight regardless of APAP treatment (Fig. 2).

**APAP Treatment Increases Serum ALT Activity in Control Mice**

Mouse serum was obtained at the time of sacrifice and used to determine the levels of ALT activity, an indicator of liver damage. It is interesting to note that although histological examination of liver sections of mice fed TMHF do not demonstrate necrosis, fibrosis or the presence of inflammatory infiltration the serum ALT levels from mice fed TMHF without APAP
treatment (0 time point) demonstrated approximately two-fold higher levels of ALT than control mice. In control mice, ALT activity peaked at 12 h following APAP treatment at levels approximately 12 fold higher than the 0 time point; ALT activity returned to base level by 24 h after APAP treatment (Fig. 3). No significant increase in ALT activity at early time points (3 or 6 h) was detected. Surprisingly, in TMHF-treated mice, there was no corresponding peak in ALT level at 12 h following APAP treatment or at any of the time points after APAP treatment. APAP treatment had a minimal effect on ALT levels in the TMHF-treated mice. The greatest increase in ALT activity in TMHF-treated mice, which occurred at 24 h after APAP treatment, was only 1.5 fold the level at the 0 time point for TMHF-treated mice and 3.0 fold the level at the 0 time point for control mice.

APAP Treatment does Not Lead to Centrilobular Vacuolation/Necrosis in TMHF-Treated Mice

H&E stained liver sections were scored according to the grading scheme described in Materials and Methods (Fig. 4; Table 1). Liver sections from TMHF-treated mice (0 h) exhibited typical hepatic architecture such as mono- and bi-nucleated hepatocytes with prominent nucleoli. Iron deposits (brown pigments) were readily detected throughout the liver tissues harvested from all TMHF-treated mice regardless of APAP treatment (Table 1). In agreement with the ALT assay results, only control mice developed pathological changes in their livers. Centrilobular vacuolation (grade 1 or 2), a typical feature of APAP toxicity, was detected in 3 of the 4 control mice at 3 h post APAP treatment (Fig. 4A). At 12 h post APAP treatment, liver sections from 3 of the 4 control mice displayed centrilobular vacuolation as well as necrosis. Prominent hemorrhage also was detected around the centrilobular area at the 12 h time point (Fig. 4A). In contrast, at the 6 hour time point, for unknown reasons, centrilobular
vacuolation was not detected in tissue sections from any of the four control APAP treated mice (Fig. 4A, Table 1). At 24 h post APAP treatment, one mouse showed centrilobular necrosis (grade 2) but the remaining mice treated with a control diet had normal hepatic morphology indicating recovery from transient APAP toxicity. In contrast, no APAP induced liver injury was observed in TMHF-treated animals at any time point (Fig. 4B, Table 1).

**APAP Treatment Induces the Formation of Protein Adducts in Mice Fed a Control Diet**

APAP is metabolically activated by the cytochrome P450 system to the highly reactive metabolite N-acetyl-p-benzoquinoneimine (NAPQI). NAPQI is then detoxified by GSH to form an acetaminophen-GSH conjugate which is not toxic. When depletion of GSH occurs at a rate that exceeds its replenishment, NAPQI covalently binds to cysteine residues on cellular proteins forming APAP protein adducts. To further evaluate APAP induced liver injury, APAP adducts were measured by immunohistochemical analysis in liver sections at 0, 3, 6, 12, and 24 h post APAP treatment. APAP adducts formed at 3 h post APAP treatment and peaked at 12 h post APAP treatment in control mice (Fig. 5A). At 24 h after APAP treatment, APAP adducts were no longer present. Positive staining for APAP adducts was exhibited in the centrilobular hepatocytes, but not in the periportal area. Western blot analysis was also used to measure APAP adducts (Fig. 5C). APAP protein adducts (approximately 60 kDa) were detected in protein from the livers of control mice. Consistent with the results of histopathological analysis, the APAP adducts began to appear at 3 h post APAP treatment and markedly increased at 12 h post APAP treatment with band intensity diminished at 24 h after APAP treatment. In contrast, APAP adducts were not detected by immunohistochemical analysis (Fig. 5B) and only barely detectable by Western blot analysis of liver protein from TMHF-treated mice (Fig. 5C).
of two representative protein samples from each control group and one from each TMHF-treated
group are shown (Fig. 5C).

In addition to the formation of APAP protein adducts, an additional characteristic of
APAP toxicity is the presence of nitrotyrosine protein adducts in the centrilobular regions of the
liver. Immunohistochemical staining and Western blot analysis were used to measure
nitrotyrosine adducts and the results were similar to that from APAP adduct analysis. Positive
staining for nitrotyrosine adducts emerged at 3 h post APAP treatment, was maximal at 12 h post
APAP treatment and disappeared at 24 h after APAP treatment in mice fed a control diet (Fig.
6A). Nitrotyrosine adducts were detected in the centrilobular area throughout the liver sections.
Two major bands (approximately 55 kDa and ≤28 kDa) in control mice were detected by
Western blot analysis for nitrotyrosine (Fig. 6C). At 12 h after APAP treatment, the protein
levels of nitrotyrosine adducts peaked. Lower levels of adducts were also detected at 3 and 6 h
post APAP treatment. Positive immunohistochemical staining for nitrotyrosine adducts was not
observed in tissue sections from any time point after APAP treatment (Fig. 6B); positive bands
for nitrotyrosine adducts also were not detected by Western blot analysis in TMHF-treated mice
(Fig. 6C). Analysis of two representative protein samples from each control group and one from
each TMHF group are shown (Fig. 6C).

Expression Levels of CYP2E1 and CYP1A2 Proteins are Not Significantly Different in TMHF-
Treated Compared to Control Mice

CYP2E1 and CYP1A2 are the major enzymes of APAP bioactivation in humans and
rodents. The effect of hepatic iron overload on both CYP2E1 and CYP1A2 protein expression
levels was determined using Western blot analysis of liver microsomes from control mice and
mice treated with TMHF for four weeks (Fig. 7). Although, the CYP2E1 and CYP1A2 protein levels in TMHF-treated animals compared to control animals were reduced by approximately 20% and 10%, respectively (Fig. 7B), the differences in CYP2E1 and CYP1A2 protein levels in TMHF-treated compared to control mice were not statistically significant.

*GSH Depletion is Markedly Greater Following APAP Treatment of Control Mice than of TMHF-Treated Mice*

Metabolism of APAP to NAPQI is accompanied by a rapid marked depletion of GSH. To address the question of the effect of TMHF treatment on APAP-induced GSH depletion, total GSH concentration in livers from control and TMHF-treated mice was measured at 0, 3, 6, 12, 24 h post APAP (300 mg/kg) treatment (Fig. 8). At 3 h after APAP treatment of control mice, the GSH level was reduced to 13% of the level prior to APAP treatment (0 h) time. By 6 h, the GSH levels recovered partially and, by 12 h, the levels had returned to 91% of the level seen prior to APAP treatment. Although APAP treatment of TMHF-treated mice led to a decrease in GSH, the magnitude was significantly less. Specifically, at 3 h after APAP treatment of TMHF-treated mice, the GSH level was only reduced to 55% of the level prior to APAP treatment and was not reduced further at the 6, 12 or 24 h time points after APAP treatment.
DISCUSSION

In this study, APAP treatment of control mice at 300 mg/kg induced transient moderate hepatotoxicity in agreement with previous studies. When the experiment was terminated at 24 h after APAP injection, none of the mice had succumbed which was the expected outcome at this APAP dose. GSH was markedly depleted to 13% of the control level at 3 h after APAP treatment. Centrilobular vacuolation/necrosis was detected in 3 of 4 mice at both the 3 and 12 h time points but not at the 6 h time point; at the 24 h time point centrilobular vacuolation/necrosis was only observed in one of the five mice sacrificed and the remaining 4 mice had normal histology. Serum ALT reached a peak level at 12 h and was markedly reduced by 24 h. Both APAP adducts and nitrotyrosine adducts were also detected transiently in APAP treated control mice.

TMHF administered in the diet has been historically used in rats as an ideal iron donor for inducing hepatic iron overload. Our laboratory reproduced these findings in mice and showed that hepatic iron levels steadily increase with TMHF feeding of mice for at least nine weeks (Isom et al., unpublished data). For the experiments in this APAP study, a time course of four weeks of THMF feeding was chosen. Mice fed an iron supplemented (THMF) diet for four weeks prior to APAP treatment developed the expected characteristics of hepatic iron overload. H&E stained liver sections from TMHF-treated mice exhibited spots of brown pigment indicating iron deposits. No pigment was detected in any of the liver tissue sections from the control mice. Strong positive staining with Perls’ Prussian blue, a specific stain for excess iron, was observed only in liver tissue sections from TMHF-treated and not in control mice providing further support that the TMHF-treated mice demonstrated iron overload. Iron regulation in the liver has been well studied. An increase in ferritin levels, one of the expected responses to
elevated hepatic iron, was clearly apparent from Western blot analysis of liver protein from TMHF-treated compared to control. Mice treated with TMHF for four weeks demonstrated hepatomegaly that has been observed previously in experimental animals and in humans with hemochromatosis.

APAP is a dose-related toxicant. The chemical APAP is not toxic, but in the liver is bioactivated by the cytochrome P450 system to the highly reactive metabolite NAPQI. CYP2E1 and CYP1A2 are the major enzymes important for APAP bioactivation in humans and rodents. At recommended doses, NAPQI is detoxified by GSH to form an acetaminophen-GSH conjugate. However, when depletion of GSH occurs at a rate that exceeds its replenishment, NAPQI covalently binds to cysteine residues on cellular proteins forming APAP-protein adducts. This metabolic phase of APAP-induced toxicity has been well known for many years. However, although formation of APAP-protein adducts correlates well with hepatotoxicity, evidence accumulated suggesting that covalent binding per se was not the mechanism of APAP toxicity. These findings lead to the concept that events downstream of GSH depletion including oxidative stress also play a critical role in APAP hepatotoxicity. The mechanism elucidated to date is complex. Glutathione depletion leads to an increase in reactive oxygen and nitrogen species in hepatocytes. Nitric oxide reacts with superoxide to form peroxynitrite which nitrates tyrosine leading to the formation of proteins with nitrotyrosine adducts. Oxidative stress leads to alterations in calcium homeostasis. Mitochondrial permeability transition results in the loss of mitochondrial membrane potential, loss of cytochrome c and decreased synthesis of ATP.

Our knowledge of how iron injures cells in the liver is by no means complete. Iron excess in hepatocytes leads to oxidative stress. In patients with HH, enhanced oxidative stress was observed as evidenced by increased oxidatively modified serum proteins and hepatic
malondialdehyde-protein adducts. Lipid peroxides have been shown to be important in the pathogenesis of HH. The main mechanism underlying progression to HCC in HH is thought to be iron-induced oxidative stress. In hepatitis C, elevated hepatic iron has been shown to be a confounding factor in many ways. The major cause of HCC in the West is chronic HCV. Disease progression of chronic HCV infection to cirrhosis and HCC most likely involves an additional risk factor to provide a second hit. It has been demonstrated experimentally in chimpanzees that HCV infection leads to an increase in body iron levels and in cell culture studies that iron alters HCV replication. Hepatic iron levels in patients with chronic hepatitis C, in particular, in individuals who have progressed to cirrhosis, can reach levels as high as are seen in individuals with HH. However, even moderate hepatic iron overload has been implicated as a fibrogenic factor leading to progressive liver disease in the setting of chronic HCV.

The effect of iron overload on hepatic mitochondrial function, initially studied in rats showed that dietary iron overload impaired mitochondrial respiration, led to lipid peroxidation of the mitochondria, caused a decrease in cytochrome c oxidase, impaired calcium sequestration by mitochondria and resulted in a decrease in hepatic ATP concentration. Studies in primary rat hepatocytes also showed that iron overload caused mitochondrial permeability transition.

Since excess iron and APAP both induce oxidative stress and mitochondrial dysfunction, we hypothesized that feeding mice a TMHF-supplemented diet would enhance APAP hepatotoxicity. The results were the opposite of what was expected. TMHF-treatment protected the mice from the transient hepatotoxicity observed after treatment with APAP at 300 mg/kg. Specifically, centrilobular vacuolation/necrosis, APAP-adducts, nitrotyrosine adducts, and a spike in serum alanine aminotransferase, which were observed in control mice treated with APAP, were not observed in TMHF-fed mice treated with APAP. These data clearly indicated
that this protection occurred prior to the formation of APAP adducts.

Therefore, we addressed the question of whether TMHF treatment alters the expression of either of two steps upstream of APAP adduct formation, specifically (1) CYP2E1 and CYP1A2 expression and/or (2) APAP induced depletion of GSH. CYP2E1 and CYP1A2 are the major enzymes important for APAP bioactivation. We examined whether TMHF fed-mice had altered CYP2E1 and CYP1A2 expression. There is precedent for this question because previous published reports show that regulation of CYP2E1 and 1A2 by specific agents can play a role in sensitivity or resistance to APAP. For example, exposure to ethanol enhances sensitivity to APAP. Consumption of alcoholic beverages is a risk factor in APAP hepatotoxicity. Alcohol consumption has been shown to induce CYP2E1 expression in experimental animals and in human hepatocytes. More recently, it has been demonstrated using Cyp2e1(-/-) mice pretreated with ethanol plus isopentanol, that CYP2E1 is not essential for enhanced APAP hepatotoxicity in alcohol treated mice; however when CYP2E1 was present, it contributed to enhanced APAP toxicity. Several agents have been shown to provide protection against APAP hepatotoxicity. When the liver is injured by APAP or other centrilobular hepatotoxicants (e.g. CCl₄), there is a recovery phase that includes increased DNA synthesis and hepatocyte proliferation. During this period, rodents are resistant to challenge with an increased dose of the same hepatotoxicant, a phenomenon called autoprotection. In one study, when mice were pretreated for 8 days with increasing doses of APAP (50 mg/g to 350 mg/kg) they developed minimal hepatotoxicity when challenged on day 9 with normally supralethal doses of APAP. Multiple mechanistic components contributed to the protection, including that the pretreatment regimen downregulated the levels of CYP2E1 and CYP1A2 in hepatic microsomes by 55% to 70% respectively. In a separate study, in which mice were pretreated with a single dose of CCl₄ (0.05 ml/kg) 24 h
before challenge with APAP (350 mg/kg), serum ALT levels were reduced by almost 90%, an example of heteroprotection. Western blot analysis showed that CYP2E1 and CYP1A2 were reduced by more than 50%. Our results showed that the levels of CYP2E1 and CYP1A2 were not significantly different in TMHF-treated compared to control mice indicating that APAP protection mediated by TMHF treatment, in contrast to what has been observed for pretreatment with APAP or CCl₄, is not caused by decreased levels of CYP2E1 and CYP1A2.

We then examined the effect of feeding mice a TMHF-supplemented diet on APAP-induced depletion of GSH. TMHF treatment had no effect on GSH level in the absence of APAP treatment. In mice fed a control diet, GSH levels were reduced by 87% at 3 h after APAP treatment, as expected. In contrast, GSH levels in TMHF-fed mice were only reduced by 45% at 3 h after APAP treatment and not reduced further at the 6, 12 or 24 h time points after APAP treatment. These findings indicate that one contributing factor to protection afforded by TMHF treatment is the ability to partially block APAP-induced depletion of GSH. The ability of pretreatment with the hepatotoxicant thioacetamide (TA) to protect rats from APAP-induced lethality, another example of APAP heteroprotection, has also been studied. When rats were treated with a low dose of TA for 36 h, TA pretreatment protected against a challenge with a high dose of APAP. The effect of TA pretreatment on APAP depletion of GSH was measured. The level of GSH was not affected by TA pretreatment alone similar to our observation for TMHF treatment. However, the level of APAP-induced GSH depletion was actually greater in TA pretreated rats than rats treated with only APAP, which was the opposite of the effect of TMHF treatment. In mice autoprotected by an 8 day pretreatment regimen of increasing APAP doses in which pretreatment led to significantly decreased CYP2E1 and CYP1A2 levels, APAP autoprotection had no effect alone on GSH levels and also had no effect on APAP-induced GSH
depletion. At 4 h after APAP challenge, 93% depletion of GSH was observed for control mice and 92% depletion of GSH for APAP pretreated mice. When APAP heteroprotection mediated by CC1₄ pretreatment was studied, the authors reported that the 24 h pretreatment with CC1₄ had no effect on GSH levels prior to APAP challenge, but the effect of CC1₄ pretreatment on APAP-induced depletion of GSH was not measured.

Studies on APAP autoprotection and heteroprotection have focused not only on the ability of the protecting agent to alter APAP bioactivation and GSH level but also have taken into consideration endogenous responses of the liver to insult including increased DNA synthesis, hepatocyte proliferation and hepatomegaly. Hepatocyte proliferation has been implicated heavily in heteroprotection by TA and as a contributing factor in APAP autoprotection. A finding known to accompany hemochromatosis in humans is hepatomegaly. Rats and mice (as reported in this study in Fig. 2), when fed a TMHF diet, develop hepatomegaly. It has also been reported that hepatic iron overload leads to increases in both cyclin D1 protein and mRNA levels in rats increased cyclin D1 expression in mice and increased PCNA labeling index. We have also observed in our studies that mice fed a TMHF supplemented diet for four weeks have an increased PCNA labeling index and increased levels of PCNA and cyclin D1 proteins as measured by Western blot (data not shown).

Autoprotection and heteroprotection from APAP hepatotoxicity are clearly mediated by different pathways depending on the agent inducing protection. We conclude that TMHF treatment protected mice from moderate transient APAP-induced hepatotoxicity, thereby acting as a heteroprotectant. Protection occurred prior to the formation of APAP-adducts. TMHF treatment had no effect on CYP2E1 or CYP1A2 levels. TMHF treatment markedly decreased the ability of APAP to deplete GSH indicating that one contributing factor to protection afforded
by feeding mice a TMHF-supplemented diet is the ability to partially block APAP-induced depletion of GSH.

We conclude that mice fed a TMHF-supplemented diet demonstrate hepatic iron overload as indicated by pigment in H&E stained liver sections and strong positive staining with Perls’ Prussian blue. TMHF-fed mice also demonstrate specific hallmarks of hepatic iron overload including hepatomegaly and increased ferritin levels. We also conclude that mice fed a TMHF-supplemented diet demonstrate protection from moderate transient APAP-induced hepatotoxicity. Taken together these findings show an association between hepatic iron overload in mice fed a TMHF-supplemented diet and protection from moderate transient APAP-induced hepatotoxicity. The conclusions of this study are limited to effects caused by feeding mice a TMHF-supplemented diet and cannot be extrapolated to the wider context of hepatic iron overload. The question will have to be addressed using independent methodologies and the findings verified before the conclusion can be reached that hepatic iron overload protects the liver from APAP-induced toxicity.
FUNDING:
This work was supported by the National Institute Diabetes and Digestive and Kidney [DK073897-01, DK073897-02S1, DK073897-01A2S1].

ACKNOWLEDGEMENTS:
We thank the Biostatistics and Organic Synthesis Core of the Penn State Hershey Cancer Institute. We also thank Thomas Miller for excellent technical assistance and assistance with manuscript preparation.
REFERENCES


Gastroenterology 101, 806-811.


Larson, A. M., Polson, J., Fontana, R. J., Davern, T. J., Lalani, E., Hynan, L. S., Reisch, J. S.,


Patten, C. J., Thomas, P. E., Guy, R. L., Lee, M., Gonzalez, F. J., Guengerich, F. P., and Yang,


Liver iron excess in patients with hepatocellular carcinoma developed on non-alcoholic steatohepatitis. *J. Hepatol.* **50**, 351-357.


**FIG. 1.** Detection of iron deposition and the ferritin protein levels in control and TMHF-treated mice. (A) Mice fed either control or TMHF diet for 4 wks were injected with 300 mg/kg APAP at various time points. Mice were sacrificed and liver tissues were harvested at each time point and fixed in buffered formalin. The fixed liver sections were stained by Perls’ Prussian blue staining and photographed by light microscopy (Original magnification: x 600). Representative data from both control and TMHF-treated mice at 0 h are shown for Perls’ Prussian blue staining. (B) A portion of the liver tissue from control and TMHF-treated mice was homogenized and subjected to Western blot analysis for ferritin and β-actin (as a loading control). Data from all four control or TMHF-treated mice at 0 h are shown for Western blot analysis.

**FIG. 2.** Effect of iron overload on liver/body weight ratio in TMHF-treated mice. Mice at the indicated time point from each group were weighed to determine total body weight, subjected to cardiac puncture. Immediately thereafter, livers were removed and weighed. Values represent the mean ± SD.
FIG. 3. Levels of serum ALT activity in control and TMHF-treated mice after APAP treatment. Serum ALT activity from mice was measured at 0, 3, 6, 12, 24 h after APAP (300 mg/kg) treatment. Results shown represent the mean ± SD per group. * P < 0.05 vs. 0 h within group, # P < 0.05 vs. control within the same time.

FIG. 4. Liver histopathology in control and TMHF-treated mice after APAP treatment. At the indicated time point after APAP treatment, control (A) and TMHF-treated mice (B) were sacrificed and liver sections were stained by H&E and photographed by light microscopy. Arrows indicate centrilobular vacuolation/necrosis (A). (Original magnification: x 600).

FIG. 5. Detection of APAP protein adducts in control and TMHF-treated mice after APAP treatment. Animals were treated with either control diet (A) or TMHF diet (B) for 4 wks and then sacrificed at the indicated time points. Liver sections were stained for APAP protein adducts using an immunohistochemical analysis. Arrows indicate positive staining for APAP protein adducts in the centrilobular area (A). (C) Livers were removed at 0, 3, 6, 12, 24 h post APAP treatment. At each time point, two representative liver homogenates from control mice and one protein homogenate from TMHF-treated mice were subjected to Western blot analysis for APAP adducts.

FIG. 6. Detection of nitrotyrosine protein adducts in both control and TMHF-treated mice after APAP treatment. Liver sections from both control (A) or TMHF-treated mice (B) were incubated with a nitrotyrosine antibody against nitrotyrosine protein adducts at various time points after APAP treatment. Arrows indicate positive staining for nitrotyrosine protein adducts.
in the centrilobular area (A). (C) Western blot analysis was performed to determine the formation of nitrotyrosine protein adducts. 20 µg of protein from two control samples and one TMHF-treated sample at each time point were loaded and analyzed.

**FIG. 7.** Protein expression of CYP2E1 and CYP1A2 in control and TMHF-treated mice. Western blot analyses for CYP2E1 and CYP1A2 were performed using microsomal proteins (10 µg/lane) from control and TMHF-treated mice. β-actin was used for a loading control (A). Protein expression levels of CYP2E1 and CYP1A2 were quantified and normalized to control mice, respectively. The data were presented as means ± SE (B). The differences in CYP2E1 and CYP1A2 protein levels in TMHF-treated compared to control mice were not statistically significant. For CYP2E1 group, $P = 0.255$; for CYP1A2 group, $P = 0.632$.

**FIG. 8.** Changes in total GSH levels in control and TMHF-treated mice. Total GSH concentration from mice was measured at 0, 3, 6, 12, 24 h post APAP (300 mg/kg) treatment. Values were means ± SE. * $P < 0.05$ vs. 0 h within group, # $P < 0.05$ vs. control within the same time.
Table 1. The Incidence and Degree of Histopathological Findings in Control or TMHF-treated Mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>0 h</th>
<th>3 h</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
<td>4/4</td>
<td>0</td>
<td>4/4</td>
<td>0</td>
</tr>
</tbody>
</table>

The incidence and degree of histopathological findings were noted according to the grading system described in the Materials and Methods. Representative sections from 4 or 5 mice per each condition were used for analysis.

“a”: Brown pigment indicates iron deposits

C: control, T: TMHF