DMD Fast Forward. Published on July 19, 2007 as doi:10.1124/dmd.107.015107

DMD #15107

EFFLUX TRANSPORTER EXPRESSION AND ACETAMINOPHEN METABOLITE EXCRETION ARE ALTERED IN RODENT MODELS OF NON-ALCOHOLIC FATTY LIVER DISEASE

Andrew J. Lickteig, Craig D. Fisher, Lisa M. Augustine,

Lauren M. Aleksunes, David G. Besselsen, Angela L. Slitt,

Jose E. Manautou, and Nathan J. Cherrington

Department of Pharmacology and Toxicology, College of Pharmacy,

University of Arizona, Tucson, AZ, 85721 (A.J.L., C.D.F., L.M.A., D.G.B., N.J.C.);

Department of Pharmaceutical Sciences, School of Pharmacy,

University of Connecticut, Storrs, CT, 06269 (L.M.A., J.E.M.);

Department of Biomedical and Pharmaceutical Sciences,

College of Pharmacy, University of Rhode Island, RI, 02881 (A.L.S.).

DMD Fast Forward. Published on July 19, 2007 as DOI: 10.1124/dmd.107.015107
This article has not been copyedited and formatted. The final version may differ from this version.

Running title:

Efflux Transporters and APAP Excretion are Altered in Rodent NASH

Corresponding author: Nathan J. Cherrington, Ph.D., Department of Pharmacology and Toxicology, College of Pharmacy, University of Arizona, 1703 East Mabel, Tucson, AZ 85721. Phone: (520) 626-0219 Fax: (520) 626-2466 Email cherrington@pharmacy.arizona.edu

Manuscript details: Text pages: 35

Figures: 12

References: 40

Words in the Abstract: 250

Words in the Introduction: 750

Words in the Discussion: 1497

Abbreviatons: APAP, acetaminophen; APAP-CG/CYS, acetaminophen-cysteinylglycine/cysteine; APAP-GLUC, acetaminophen-glucuronide; APAP-GSH, acetaminophen-glutathione; APAP-NAC, acetaminophen-mercapturate (*N*-acetyl-L-cysteine); APAP-SULF acetaminophen-sulfate; Bcrp, Breast cancer resistance protein; bDNA, branched DNA; Bsep, Bile salt excretory protein; Gapdh, Glyceraldehyde 3-phosphate dehydrogenase; GST, glutathione *S*-transferase; HF, high-fat; IL, Interleukin; MCD, methionine-, choline-deficient; Mrp, Multidrug resistance-associated protein; NAFLD, Non-alcoholic Fatty Liver Disease; NAPQI, *N*-acetyl-*p*-benzoquinoneimine; NASH, non-alcoholic steatohepatitis; RLU, relative light units; RT, room temperature; SEM, standard

DMD Fast Forward. Published on July 19, 2007 as DOI: 10.1124/dmd.107.015107
This article has not been copyedited and formatted. The final version may differ from this version.

error of the mean; SFL, Simple Fatty Liver.

DMD Fast Forward. Published on July 19, 2007 as DOI: 10.1124/dmd.107.015107
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

Efflux transporters are responsible for the excretion of numerous xenobiotics and endobiotics and thus play an essential role in proper liver and kidney function. Non-alcoholic fatty liver diseases (NAFLD) comprise a spectrum of disorders that range from simple fatty liver (SFL) to non-alcoholic steatohepatitis (NASH). While the precise events leading to NAFLD are unclear, even less is known about the effects on efflux transporter expression and drug disposition. The purpose of this study was to determine the effect of NAFLD on efflux transporter expression in rat liver, as well as acetaminophen (APAP) metabolite excretion. To simulate SFL and NASH, rats were fed either a high-fat (HF) or a methionine-, choline-deficient (MCD) diet for 8 weeks. In the livers of MCD rats, there were striking increases in both mRNA and protein levels of Mrp3, Mrp4 and Bcrp, as well as increased Mrp2 protein. Following administration of a non-toxic dose of APAP, biliary concentrations of APAP-sulfate, APAP-glucuronide (APAP-GLUC) and APAP-glutathione were reduced in MCD rats. The effects of the HF diet on both transporter expression and APAP disposition were by comparison far less dramatic than the MCD diet-induced alterations. While APAP-sulfate levels were also decreased in MCD rat plasma, the levels of the Mrp3 substrate APAP-GLUC were elevated. Urinary elimination of APAP metabolites was identical between groups, except for APAP-GLUC, the concentration of which was 80% higher in MCD rats. These studies correlate increased hepatic Mrp3 protein in the MCD model of NASH with increased urinary elimination of APAP-GLUC. Furthermore, the proportional shift in elimination of APAP metabolites from bile to urine

indicates that MCD-induced alterations in efflux transporter expression can affect the route of drug elimination.

INTRODUCTION

Non-Alcoholic Fatty Liver Disease (NAFLD) comprises a spectrum of pathologic lesions that ranges from hepatic steatosis to a form of fatty liver hepatitis known as non-alcoholic steatohepatitis (NASH). Histologic features independently associated with the diagnosis of NASH in human biopsies include hepatic steatosis, hepatocyte ballooning, lobular inflammation, Mallory's hyaline and perisinusoidal fibrosis. Moreover, NASH has the potential to advance to cirrhosis requiring liver transplant (Abdelmalek et al., 1995;Caldwell and Hespenheide, 2002;Charlton et al., 2001;Powell et al., 1990). A recent overview of clinical data estimates that NAFLD and NASH affect 17-33% and 5.7-17% of American adults, respectively. Although the etiology of NASH is not completely understood, most investigators agree that a critical baseline of steatosis requires a second "hit" of oxidative stress that promotes the subsequent inflammation and fibrosis of NASH. NAFLD is primarily associated with features of the metabolic syndrome, including obesity, diabetes, dyslipidemia, and insulin resistance (McCullough, 2006). Importantly, these NAFLD-associated features invariably require pharmacologic intervention with one or more therapeutic agents. It therefore follows that a significant portion of the NAFLD demographic is already using prescription medication.

In the present study, NAFLD was recapitulated in two of its prominent forms, simple fatty liver (SFL) and NASH, respectively. The SFL model utilized a high-fat (HF) diet, while the NASH model utilized a methionine-, choline-deficient

(MCD) diet. The histologic changes that occur in the MCD model have been thoroughly characterized and determined to be remarkably similar to those seen in human NASH (George et al., 2003). Histologic features of NASH necessarily include hepatic steatosis, mild, lobular inflammatory infiltration, and hepatocellular ballooning degeneration (Brunt and Tiniakos, 2005a).

Because the vast majority of drug metabolism occurs in the liver, it is particularly important to understand the transport processes involved in the elimination of drug metabolites formed in the organ, especially a diseased one. In the liver, there are two basic types of transporters. These include the uptake transporters, e.g. Organic anion-transporting polypeptides (Oatps) and Organic cation transporters (Octs), which facilitate the extraction of drugs from portal blood into hepatocytes, and the efflux transporters, e.g. Multidrug resistance-associated proteins (Mrps), Bile salt excretory protein (Bsep) and Breast cancer resistance protein (Bcrp), which extrude their substrates out of hepatocytes into bile or blood. While not all drugs require uptake transport to cross the sinusoidal membrane into hepatocytes, virtually all of their metabolites require efflux transporters in order to be excreted into bile and blood for biliary and urinary elimination, respectively.

Acetaminophen (APAP) provides a fine example of the integral role played by efflux transporters in drug metabolite excretion from liver. Following passive diffusion into hepatocytes, APAP is mainly conjugated with sulfate (SULF) or

glucuronic acid (GLUC) to form APAP-SULF and APAP-GLUC, respectively (Moldeus, 1978;Grafstrom et al., 1979). A small portion of a dose of APAP will undergo cytochrome P450 metabolism to the reactive metabolite *N*-acetyl-*p*-benzoquinoneimine (NAPQI), which is subsequently conjugated with glutathione (GSH) to form APAP-GSH. In rats, excretion of APAP-GLUC and APAP-GSH metabolites from the liver occurs predominantly via the biliary route, whereas the APAP-SULF metabolite predominantly undergoes sinusoidal efflux and subsequent elimination in the urine (Gregus et al., 1988c). Importantly, all APAP metabolites require efflux transport in order to be excreted from the liver, and each can be detected in both bile and urine (Chen et al., 2003b;Gregus et al., 1988b;Xiong et al., 2002b;Zamek-Gliszczynski et al., 2006a;Manautou et al., 2005b).

In vivo disposition studies and *in vitro* functional transport experiments indicate Mrp2, Mrp3, Mrp4 and Bcrp each have the ability to transport a variety of unconjugated and conjugated drugs, including APAP metabolites (Buchler et al., 1996;Keppler et al., 1997;Nakanishi et al., 2003;Xiong et al., 2000;Zamek-Gliszczynski et al., 2005a;Zamek-Gliszczynski et al., 2006b). It is important to emphasize the distinctive location of these four transporters in hepatocytes, as well as their respective APAP metabolite substrates. Mrp2 and Bcrp are localized to the canalicular (apical) membrane of hepatocytes from which they excrete their substrates into the bile canaliculi. Accordingly, in a healthy liver, biliary excretion of the SULF, GLUC and GSH conjugates of APAP is predominantly

mediated by Mrp2, while Bcrp appears also to contribute to excretion of APAP-SULF conjugates (Borst et al., 2000;Buchler, Konig, Brom, Kartenbeck, Spring, Horie, and Keppler, 1996;Chen et al., 2003a;Keppler and Konig, 1997;Keppler et al., 2000;Keppler and Konig, 2000;Zamek-Gliszczynski et al., 2005b). Mrp3 and Mrp4 are expressed at the sinusoidal (basolateral) membrane of hepatocytes and cholangiocytes from which they expel their substrates into the blood (Konig et al., 1999;Donner and Keppler, 2001;Soroka et al., 2001). Sinusoidal excretion of the APAP-GLUC metabolite from hepatocytes is predominantly mediated by Mrp3, while Mrp4 appears to mediate excretion of APAP-SULF metabolites (Chen et al., 2001;Zamek-Gliszczynski et al., 2006d;Manautou et al., 2005a). Recent studies indicate that Mrp3 and Mrp4 have an equal role in the efflux of APAP-SULF (Zamek-Gliszczynski et al., 2006c).

During other liver disease states such as septic cholestasis, extrahepatic cholestasis and alcoholic and viral hepatitis, human and rodent studies have demonstrated alterations in cytochrome P450-mediated drug metabolism, transport and pharmacokinetics (Congiu et al., 2002;Farrell et al., 1978;Farrell et al., 1979;Figg et al., 1995;Forrest et al., 1977;Kubitz et al., 1999;Nadai et al., 2001;Narang et al., 1985;Narang et al., 1982;Narang et al., 1981;Westphal and Brogard, 1997). However, there is little information on the effect of NAFLD on these same parameters. Therefore, the purpose of the present study was to determine the effect of NAFLD on efflux transporter expression in liver, as well as disposition of APAP metabolites.

METHODS

Materials. APAP and urethane were purchased from Sigma-Aldrich (St. Louis, MO). The analytical column and guard cartridges for high performance liquid chromatography (HPLC) analysis were purchased from Bodman (Aston, PA). HPLC-grade water, HPLC-grade methanol, Tris base, sodium dodecyl sulfate, sodium chloride, and sucrose were purchased from Fisher Scientific (Pittsburgh, PA), and all other chemicals used were of reagent grade or better.

Treatment of animals. Male Sprague-Dawley rats weighing 200 to 250 g were purchased from Harlan Laboratories Inc. (Bar Harbor, ME). Animals were housed in a temperature-, light-, and humidity-controlled environment in hanging cages with hardwood chips. Rats (n = 5–9/group) were randomly placed into one of three diet treatment groups: control group to simulate healthy liver (Teklad 7001 4% Mouse/Rat Diet; Madison, WI), HF group to simulate SFL [18% butter (w/w) diet] and MCD group to simulate NASH (methionine-, choline-deficient diet) (Dyets Incorporated; Bethlehem, PA) ad libitum for 8 weeks. According to its manufacturer, the Teklad 7001 4% Mouse/Rat Diet is a “complete and balanced, fixed formula diet formulated to provide consistent nutrition to research rodents.” The Institutional Animal Care and Use Committee of the University of Arizona approved the animal studies described below.

Histology. Sections of liver from control, SFL and NASH rats were embedded in Tissue-Tek® OCT compound (Electron Microscopy Sciences; Hartfield, PA),

rapidly frozen in ethanol cooled with dry ice and stored at -80º C until use. Using a Microm HM 550 cryostat (Richard Allen Scientific, Kalamazoo, MI), 5-µm slices from each diet group were generated and mounted onto Superfrost Plus slides (Fisher Scientific, Houston, TX). The mounted tissues were stained with hematoxylin and eosin (H & E) or Masson's trichrome stain. Histologic analyses were conducted under light microscopy (x40 objective) using coded slides to avoid observer bias. Parameters of the histologic analyses included degree of steatosis, inflammatory infiltration and fibrosis.

APAP excretion experiments. Under urethane-induced anesthesia (1.1 g/kg, 5 mL/kg, intraperitoneally), the left femoral artery and right femoral vein were cannulated with PE 50 polyethylene tubing. The bile duct was subsequently cannulated with PE 10 polyethylene tubing distal to the bile duct bifurcation. Before injection of APAP, the femoral artery and vein cannula were flushed with approximately 0.5 mL of saline-5% mannitol solution containing 50 units of heparin. APAP, dissolved in saline containing 5% mannitol, was injected into the femoral vein cannula at a dosage of 1 mmol/kg (10 mL/kg) using a 3-cc syringe with a 23-gauge needle. After APAP injection, the femoral vein cannula was flushed with a 0.5-mL injection of 0.5% saline-5% mannitol solution. At 0, 2, 10, 20, 40, 60, and 90 minutes after APAP administration, approximately 250-µL blood samples were collected from the femoral artery in heparinized tubes. Bile was collected at 15, 30, 45, 60, 75, and 90 minutes after APAP administration. To maintain urine flow, 1 mL/kg saline-5% mannitol solution was injected through the

femoral vein cannula every 15 minutes after APAP administration. Urine was collected from the bladder at 90 minutes after APAP administration by ligation of the bladder and removal with a 22-gauge needle and 3-cc syringe. Livers and kidneys were also harvested at the end of each APAP disposition experiment and snap frozen in liquid nitrogen. All samples were stored at -80°C until analysis.

Detection of APAP and its metabolites in bile, plasma and urine samples. APAP and its metabolites in bile, plasma, and urine samples were quantified by high performance liquid chromatography analysis based on previously described methods (Howie et al., 1977a;Chen et al., 2000;Slitt et al., 2003d). APAP and its metabolites were resolved using a Zorbmax SB-C_{18} reverse-phase 4.6-mm x 25-cm column with a Phenomenex Security Guard Column Guard and eluted using a mobile phase composed of 12.5% HPLC-grade methanol, 1% acetic acid, and 86.5% water, run isocratically at a flow rate of 1.2 mL/minute. The elution of metabolites was monitored at a wavelength of 254 nm. Retention times of APAP and its metabolites were determined by comparison with that of authentic standards. Since this HPLC method does not separate the cysteinylglycine and cysteine conjugates of APAP, they were quantified together as APAP-CG/CYS.

Samples were analyzed using a Beckman System Gold HPLC system (Beckman Coulter, Inc., Fullerton, CA) equipped with a 128-nm solvent module and a 166-nm detector. Quantitation was based on integrated peak areas. The

concentrations of APAP and its metabolites were calculated using an APAP standard curve since the molar extinction coefficients of APAP and its conjugated metabolites are approximately the same (Howie et al., 1977b). To precipitate proteins in bile, plasma, and urine, samples were diluted 1:2, 1:2, and 1:3, respectively, with ice-cold methanol and centrifuged at 4,000 x g for 30 minutes at 4°C. The resulting supernatants were collected and diluted in mobile phase 1:3 (bile and urine) or 1:2 (plasma) prior to HPLC analysis. Liver samples were homogenized (1:9 w/v) in ice-cold HPLC grade methanol. Homogenates were centrifuged at 4,000 x g for 20 minutes at 4°C, and the supernatants were filtered through a 0.45 µm filter. Samples were further diluted 1:3 with mobile phase prior to HPLC analysis.

RNA extraction. Total RNA from liver and kidney tissue was extracted using RNAzol B reagent (Tel-Test Incorporated; Friendswood, TX) according to the manufacturer's protocol. The quality of RNA samples was judged by the integrity and relative ratio of 28S and 18S rRNA bands following agarose gel electrophoresis.

Messenger RNA expression analysis. Rat Mrp1-Mrp7, Bsep and Bcrp mRNA levels were measured using the branched DNA signal amplification assay. To measure the levels of Mrp1-Mrp6, Bsep and Bcrp mRNA, respectively, oligonucleotide probe sets containing multiple mRNA transcript-specific capture, label, and blocker probes were designed using ProbeDesigner software v1.0

(Bayer Corporation-Diagnostics Division). The probe sets used in this study have been described previously, including Mrp1-3 (Cherrington et al., 2002), Mrp4-6, Bsep, Mrp7 and Bcrp (Tanaka et al., 2005b). Probes were designed with an annealing temperature of approximately 63°C, which enabled the hybridization conditions to be held constant (i.e., 53°C) during each hybridization step and for each probe set. Every probe developed in ProbeDesigner was submitted to the National Center for Biotechnological Information for nucleotide comparison by the basic logarithmic alignment search tool (BLASTn), to ensure minimal cross-reactivity with other known rat sequences and expressed sequence tags. Oligonucleotides with a high degree of similarity (≥80%) to other rat gene transcripts were excluded from the design.

Total RNA (1 µg/µL; 10 µL/well) was added to each well of a 96-well plate containing capture hybridization buffer and 50 µL of each diluted probe set. For each gene, total RNA was allowed to hybridize to the probe set overnight at 53°C. Subsequent hybridization and quantitation steps were performed according to the manufacturer's protocol (QuantiGene Reagent System, Panomics; Fremont, CA).

Western Blot Analysis of Mrp2, Mrp3, Mrp4 and Bcrp Protein Expression. Liver crude membrane preparations were made as described previously (Slitt et al., 2003c). Liver and kidney protein concentrations were determined using the BCA™ Protein Assay Kit (Pierce Biotechnology; Rockford, IL). Liver (40 µg/lane) membrane proteins were electrophoretically resolved on tris-glycine, SDS-

DMD Fast Forward. Published on July 19, 2007 as DOI: 10.1124/dmd.107.015107
This article has not been copyedited and formatted. The final version may differ from this version.

polyacrylamide gels. Proteins were transblotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were blocked for 1 hour in 5% nonfat dry milk in TBS-Tween buffer before incubation with antibodies. Primary antibody clones, membrane incubation times and dilutions in 5% nonfat dry milk were as follows. For Mrp2, conditions included a 1:2000 dilution of mouse anti-human M_2III-6 monoclonal antibodies (ID Labs Inc.; London, ON, Canada). For Mrp3, conditions included a 1:5000 dilution of affinity-purified antibodies against rat Mrp3 protein (Slitt et al., 2003b). For Mrp4, conditions included a 1:500 dilution of goat anti-human polyclonal antibodies (Abcam, Inc.; Cambridge, MA). For Bcrp, conditions included a 1:500 dilution of mouse anti-human bxp-21 monoclonal antibodies (Kamiya Biomedical Company; Seattle, WA). Blots were subjected to four 15-minute washes in TBS-Tween buffer prior to incubation for 1 hour at RT with secondary antibody. The secondary antibody dilutions included 1:2000 (Mrp2), 1:5000 (Mrp3 and Mrp4) and 1:3000 (Bcrp). Protein-antibody complexes were detected using ECL Western blotting reagents (Amersham Biosciences Inc.; Piscataway, NJ) and Blue Lite Autoradiography Film (ISC BioExpress; Kaysville, UT).

Statistics. For excretion studies, statistical differences between diet groups at each time point were determined using a one-way analysis of variance followed by a Duncan's Multiple Range post hoc test. For mRNA and protein expression analyses, statistical differences between diet groups were also determined using

a one-way analysis of variance followed by Duncan's Multiple Range post hoc test.

DMD Fast Forward. Published on July 19, 2007 as DOI: 10.1124/dmd.107.015107
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

Liver histology. Figure 1 demonstrates the effect of the respective high-fat and MCD diets on liver histology after 8 weeks. Hepatic steatosis was apparent at 2 weeks in rats fed the MCD diet but not in rats fed the control standard diet. Notably, inflammation and fibrosis were plainly absent in the MCD group at this early time-point (data not shown). However, following 8 weeks on the diet, histologic analyses of H & E- (A-C) and trichrome-stained sections (D-F) were indicative of two distinct NAFLD pathologies, such that corresponded satisfactorily with simple hepatic steatosis and early-stage NASH in human patients (Brunt and Tiniakos, 2005c). Histology of H&E-stained liver sections from the control group was unremarkable, with minimal periportal inflammatory infiltrates (A) and minimal fibrosis (D). Sections from the high-fat diet group were characterized by predominantly periportal, microvesicular steatosis (B) and minimal fibrosis (E). Sections from the MCD diet group revealed diffuse, predominantly macrovesicular steatosis (C) with mild biliary oval cell hyperplasia, mild periportal inflammatory infiltrates and fibrosis (F).

Efflux transporter mRNA levels in the liver following 8 weeks of feeding on the respective high-fat and MCD diets. Following 2 weeks of feeding on the control and MCD diets, there were no discernible differences in mRNA levels of Mrp2, Mrp3 Mrp4 or Bcrp (data not shown). This suggests that acute dietary deficiency of methionine and choline does not alter transcriptional regulation of these four major efflux transporters. Figure 2 illustrates the effect of 8 weeks of

feeding on the respective high-fat and MCD diets have on efflux transporter mRNA levels in liver. In high-fat rats, Mrp5 mRNA levels were increased 350%, whereas Mrp6 and Bsep were each decreased by 50%. Mrp1-4 and Bcrp mRNA levels were unchanged in livers of high-fat rats. In MCD rats, Mrp3, Mrp4 and Bcrp mRNA expression levels were increased 2990%, 405% and 59%, respectively. Mrp1 and Mrp2 mRNA levels were not altered by the high-fat or MCD diet.

Efflux transporter protein levels in liver following 8 weeks of feeding on the respective high-fat and MCD diets. Figure 3 shows the effect of the respective high-fat and MCD diets on Mrp2, Mrp3, Mrp4 and Bcrp protein levels in liver. In MCD rats, Mrp2, Mrp3, Mrp4 and Bcrp protein expression levels were increased 185%, 253%, 246% and 71%, respectively. Notably, no changes in protein levels of the major APAP metabolite transporters were observed in the livers of high-fat rats.

Effect of high-fat and MCD diets on bile flow. Figure 4 shows the cumulative amount of bile collected during the 90 minutes following APAP administration for control, high-fat and MCD rats. The cumulative bile volume in high-fat rats was significantly greater than the control by 19% at 60 minutes and 26% at 90 minutes. MCD rat bile flow did not differ from the control. Importantly, the data from these models of NAFLD do not indicate impairment of bile secretory function.

Effect of high-fat and MCD diets on biliary excretion of APAP and APAP metabolites. Figure 5 (***upper left panel***) shows the concentration of APAP and its major metabolites in bile over 90 minutes. The concentration of APAP in the bile of MCD rats was significantly elevated in comparison to control (20% higher) at 15 minutes. It is important to note that parent APAP that does not undergo metabolism within a hepatocyte can passively diffuse across the canalicular or basolateral membrane. Thus, any inter-group differences in the biliary or urinary concentration of APAP would not be reflective of alterations in function by efflux transporters.

The biliary concentration of each major metabolite of APAP was recurrently observed to be significantly decreased in MCD rats relative to control and high-fat rats. In contrast to APAP parent, each of these metabolites, i.e. APAP-GLUC, APAP-GSH and APAP-SULF, does require active transport in order to be excreted from hepatocytes.

Figure 5 (***upper right panel***) illustrates the concentration of APAP-GLUC in bile of control, high-fat and MCD groups over the 90-minute collection period. APAP-GLUC was significantly lower in MCD rats than in control throughout the 90 minutes, with the maximal difference (73% lower) being observed by 45 minutes. The biliary APAP-GLUC levels in MCD rats became significantly lower than the high-fat group by 30 minutes. Of the three major metabolites measured in the

bile, APAP-GLUC was the most abundant metabolite detected throughout the time-course in each group.

Figure 5 (***lower left panel***) illustrates the concentration of APAP-SULF in bile of control, high-fat and MCD rats over the 90-minute collection period. APAP-SULF was significantly lower in MCD rats than in control throughout the 90 minutes, with the maximal difference (67% lower) being observed by 30 minutes. In addition, the biliary APAP-SULF levels in MCD rats also became significantly lower than the high-fat group by 15 minutes. The biliary concentration of APAP-SULF in high-fat rats was lower than the control (by 23%) only at 15 minutes.

Figure 5 (***lower right panel***) illustrates the concentration of APAP-GSH in bile of control, high-fat and MCD groups over the 90-minute collection period. In the MCD group, APAP-GSH levels in bile were significantly lower than the control group throughout the 90 minutes. The maximal difference from the control group (93% lower) was observed by 30 minutes.

Effect of high-fat and MCD diets on APAP and APAP-GLUC metabolite concentration in liver. Figure 6 shows the concentration of APAP and its APAP-GLUC metabolite in the livers of control, high-fat and MCD rats, respectively, at the completion of the study. The APAP parent concentration in the livers of high-fat rats was 85% lower than the control. Notably, the concentration of APAP-GLUC in the livers of MCD rats was 79% lower than the control. The APAP-GSH,

APAP-SULF and APAP-CG/CYS metabolites were not detectable in livers in any group.

Effect of high-fat and MCD diets on plasma concentration of APAP and APAP metabolites. Figure 7 shows the concentrations of APAP, APAP-GLUC and APAP-SULF in plasma over 90 minutes. APAP-GSH was not detectable in the plasma of rats in these studies. For APAP (***top panel***), there were no plasma level differences between the control, high-fat and MCD groups throughout the time-course. The APAP-GLUC metabolite (***middle panel***) was detected in the plasma of the control and high-fat groups by 10 minutes following APAP administration. In contrast, in the MCD group, the APAP-GLUC metabolite was present in the plasma within 2 minutes of APAP administration. It is interesting that by 10 minutes, the concentration of the APAP-GLUC metabolite in the MCD animals was 4.8-fold higher than in controls. Plasma levels of APAP-GLUC in MCD rats remained significantly elevated above the control group throughout the remainder of the time-course by an average of 3.3-fold. APAP-SULF was present in the plasma of control, high-fat and MCD rats within 10 minutes of administration of the APAP (***lower panel***). Within 40 minutes through 90 minutes, the plasma concentration of APAP-SULF in MCD rats was significantly lower than the control group by an average of 50%.

Effect of high-fat and MCD diets on urinary excretion of APAP and APAP metabolites. Figure 8 shows the concentration of APAP and its metabolites in

the urine of control, high-fat and MCD rats at 90 minutes following APAP administration. There were no group differences for APAP, APAP-SULF, APAP-CG/CYS and APAP-NAC. Nor were there differences in the net (i.e. TOTAL) concentration of metabolites between control, high-fat and MCD rats. However, the concentration of the APAP-GLUC metabolite was 80% higher in the urine of MCD rats compared to the control. Importantly, this same metabolite was elevated in the plasma of MCD rats throughout the 90-minute time-course. APAP-GSH was not detected in the urine of any group.

DISCUSSION

The current study presents novel data that demonstrates the effects of experimental NAFLD on transporter expression and drug disposition. The clinical relevance of these topics is underscored by the fact that the NAFLD patient population, a demographic that uses a great quantity and variety of prescription medications, is expected to continue growing at an alarming rate (Kopelman, 2000;Brunt and Tiniakos, 2005b). In order to expand our comprehension and/or potentially develop pharmacologic treatment for this disorder, extensive study of accurate animal models will serve as an invaluable tool. It is thus pertinent to note that the current models of NAFLD (i.e. high-fat and MCD diets) provided accurate histologic representations of simple fatty liver (SFL) and non-alcoholic steatohepatitis (NASH), respectively.

It is important to note that there were no alterations in bile flow between the control and modified diet groups. The cumulative bile flow was significantly elevated in high-fat rats but only at the later time-points. These are each a contrast to the obese Zucker rat (a model of insulin resistance and obesity) in which bile secretory function in fatty liver is impaired, nonetheless in the absence of cholestatic injury (Pizarro et al., 2004a;Geier et al., 2005).

This is the first study to examine liver efflux transporter expression in a high-fat and MCD dietary models of simple hepatic steatosis and NASH in rats. Both models resulted in multiple alterations in liver and kidney expression of efflux

transporters. It is interesting to note that, when the mRNA levels of a given efflux transporter were altered in the high-fat diet, the MCD model generally generated the same effect (i.e. increased Mrp5 in liver and kidney; decreased Mrp6 in liver and kidney). In contrast, only MCD rats were characterized by up-regulation of mRNA and protein levels of the hepatic efflux transporters Mrp2, 3, 4 and Bcrp.

The obvious alterations in transporter expression and drug disposition in the current study encourage further investigation into mechanisms of transcriptional regulation during NAFLD, such that would likely provide greater insight into necessary differences at the molecular level between simple fatty liver and steatohepatitis in rats. It is useful to note that, at the histologic level, the major principal difference between the high-fat and MCD rats was the presence of inflammatory infiltrates and fibrosis in MCD rat livers. Thus, one important factor to consider with regard to efflux transporter regulation during NAFLD is the role of pro-inflammatory cytokines. Elevated levels of the pro-inflammatory cytokines Tumor necrosis factor (Tnf)-α and Interleukin (IL)-6 have been detected in the plasma of biopsy-proven NASH patients but not in patients with simple hepatic steatosis (Abiru et al., 2006;Kugelmas et al., 2003;Bahcecioglu et al., 2005). Consistent with the current model, the early stages of MCD-induced NASH are characterized by up-regulation of hepatic IL-6 mRNA and elevated plasma levels of Tnf-α (Chawla et al., 1998;Starkel et al., 2003). While there is little information on the role of IL-6 in the regulation of hepatic transporters in rats, the role of Tnf-α in this case has been well-studied utilizing the endotoxin-induced model of

sepsis (Whiting et al., 1995;Green et al., 1996;Geier et al., 2003b;Cherrington et al., 2004). Specifically, down-regulation of Mrp2 mRNA during endotoxemia has been demonstrated to be dependent on Tnf-α (Geier et al., 2003a). In contrast, in obese Zucker rats, which develop elevated plasma levels of Tnf-α, abrogation of Tnf-α activity with etanercept has no effect on Mrp2 protein levels (Liu et al., 2002;Pizarro et al., 2004b). While there is limited data on the hepatic and serum levels of pro-inflammatory cytokines in rat models of NAFLD, it seems reasonable to propose that cytokines may be important in transporter regulation in the MCD model of NASH.

Importantly, the biochemical mechanisms by which the high-fat and MCD diets induce their respective pathologies in the liver are markedly distinct. Thus, direct comparison of the effects, e.g. gene expression, between the high-fat and MCD groups was deemed inappropriate for statistical analysis in these studies. In the high-fat dietary model, induction of hepatic steatosis is most likely a consequence of increased net flux of lipids through the liver. In contrast, the MCD model achieves its histologic recapitulation of NASH via a series of events consistent with lipid peroxidation as a pathogenic mechanism for initiation and perpetuation of liver injury, inflammatory recruitment, stellate cell activation and fibrogenesis (George et al., 2003). While the initial steatosis of the MCD model is not associated with the peripheral insulin resistance that is frequently observed in human NASH, the consequent damage, including hepatocellular injury,

inflammatory recruitment and zone 3 fibrosis does resemble that observed in human NASH (George et al., 2003).

It is pertinent to note that the difference between the “control” and MCD diets utilized in the current study was not exclusive to the presence or absence of methionine and choline. An arguably more appropriate dietary control would be achieved by merely re-supplementing the MCD diet with methionine and choline. Nonetheless, both diets applied in the current study are, in fact, fully defined in terms of their amino acid content, and it is thus known that the “control” diet (Teklad 7001 4% Mouse/Rat Diet) is methionine and choline sufficient, resulting in a histology reflective of a healthy liver (Figure 1). At the same time, there is no question that the methionine and choline deficiency is responsible for the pathology observed (Figure 1F). However, it is not entirely clear whether the alterations in transporter expression (and thus APAP-GLUC metabolite excretion) are the consequence of a chronic dietary deficiency in methionine and choline, or, conversely, a regulatory response to the progressive damage and/or steatosis that occur as a result of the deficiency. According to the mRNA and histologic analysis of control and MCD rat livers following 2 week of the respective diets, acute biochemical deficiency of methionine and choline is clearly not responsible for inducing alterations in transporter expression.

The latter part of the current study examined the disposition of acetaminophen metabolites during NAFLD. In high-fat rats, there were no significant differences

in the concentration of APAP metabolites in the plasma or urine and relatively few differences in the biliary excretion of metabolites. It is therefore apparent that simple hepatic steatosis has no discernible effect on the disposition of APAP metabolites. In contrast, there were obvious alterations in the disposition of the APAP-GLUC, APAP-GSH and APAP-SULF metabolites in MCD rats.

One of the most important observations in the current study was the altered disposition of APAP-GLUC metabolites in the rats with MCD-induced NASH. The marked reduction in the biliary concentration of APAP-GLUC was accompanied by a concurrent increase in its plasma and urine concentrations. These were in the range of those observed following pretreatment of the same strain of rats with various Mrp3-inducing compounds, including phenobarbital (Brouwer and Jones, 1990b;Slitt et al., 2003a;Xiong et al., 2002a). In hepatocytes of normal rat liver, Mrp3 protein is expressed at much lower levels than Mrp2. Thus, although Mrp3 has a much higher affinity for APAP-GLUC, this metabolite is normally excreted predominantly into bile in rats (Brouwer and Jones, 1990a;Gregus et al., 1990). However, in the livers of rats with MCD-induced NASH, the expression levels of Mrp2 and Mrp3 appear to have become relatively equal, likely resulting in competition for the APAP-GLUC substrate. It therefore follows that the apparent shift from biliary to urinary excretion is most likely due to (1) the increased Mrp3 protein levels in liver and (2) the higher affinity of Mrp3 for the APAP-GLUC metabolite.

The concentration of APAP-SULF in bile was significantly lower in MCD rats compared to the control throughout the entire time-course. This result was unanticipated given the substantial up-regulation of both Mrp2 and Bcrp, which are responsible for the canalicular excretion of APAP-SULF. However, the plasma concentration of APAP-SULF was also consistently lower in MCD rats, and although the decrease of APAP-SULF in MCD rat urine did not reach statistical significance, it too appears to be slightly lower than the control. Therefore, it would seem that the differences in disposition may have resulted from a decrease in sulfotransferase expression, sulfotransferase activity, and/or the sulfotransferase cofactor 3'-phosphoadenosine-5'-phosphosulfate.

The concentration of APAP-GSH in bile was also decreased in MCD rats throughout the time-course. This result was also unforeseen, given the pronounced induction of Mrp2 protein levels. In contrast, the concentration of APAP-GSH derivatives APAP-CG/CYS and APAP-NAC in urine were not altered in MCD rats. These data indicate that sinusoidal efflux transporters responsible for the hepatovascular excretion of APAP-GSH and its derivatives were able to maintain this function in MCD rat livers. However, it is also clear that fewer APAP-GSH metabolites and derivatives were formed in MCD rat livers. This effect may be partly due to the reduction ex vivo in cytochrome P450-mediated bioactivation of APAP to the reactive NAPQI form (data not shown), which undergoes a spontaneous electrophilic reaction with GSH to form APAP-GSH. Hepatic GSH levels were not altered in either model of NAFLD (data not shown).

The metabolic capacity of the kidneys to form derivatives of the APAP-GSH conjugate is also an important consideration in the case of APAP disposition. Following efflux of APAP-GSH conjugates from the liver into the sinusoidal blood, they are rapidly degraded into APAP-CG, APAP-CYS and APAP-NAC by the kidneys (Moldeus, 1978). Thus, only the degradation products of APAP-GSH, including APAP-CG, APAP-CYS and APAP-NAC, are normally detectable in the urine (Gregus et al., 1988a). Mrp2, which is expressed in the brush border membrane of rat proximal tubule cells, is presumably responsible for the tubular secretion of these APAP-GSH derivatives (Schaub et al., 1997). There were no alterations in the urinary excretion of the APAP-GSH derivatives. Bcrp and Mrp4 are also present at high levels in rat kidneys and appear to be important in the secretion of sulfate conjugates from proximal tubule cells (Jonker et al., 2002;Mizuno et al., 2004;Tanaka et al., 2005a). In contrast, Mrp3 is expressed at the basolateral membrane of renal proximal tubule cells and may participate in efflux of APAP-GLUC into blood (Kuroda et al., 2004). Although Mrp3, Mrp4 and Bcrp are each expressed to a considerable extent in the kidney, their contribution to renal excretion of APAP-GLUC (by Mrp2) and APAP-SULF (by Mrp4 and Bcrp) is likely very limited. This is because, in rats and humans, the vast majority of APAP biotransformation, including sulfation, glucuronidation and glutathione conjugation, occurs in the liver, where sulfotransferase, UDP-glucuronosyl transferase and GST activities make their greatest contribution to drug

metabolism (Levy and Yamada, 1971;Slattery and Levy, 1979;Galinsky and Levy, 1981;Lohr et al., 1998;Vendemiale et al., 1996).

In summary, we report that diet-induced models of simple fatty liver steatosis and NASH produce multiple changes in efflux transporter expression in rats. Importantly, the MCD dietary model of NASH resulted in decreased biliary excretion of APAP-GLUC, APAP-SULF and APAP-GSH. Furthermore, the MCD model of NASH altered the vectorial excretion of APAP-GLUC, shifting excretion from bile-to-blood instead of blood-to-bile. This shift in vectorial excretion of APAP-GLUC correlated with induction of the basolateral efflux transporters Mrp3 and Mrp4. Thus the current study provides proof of principle that changes in drug transporter expression during liver disease, i.e. NASH, can result in profound alterations in the drug disposition process. More recent studies also indicate that the therapeutic efficacy of drugs is likewise integrally linked to function of drug transporters, which can distribute pharmacologically active concentrations of the drug to its target site, e.g. ezetimibe and morphine 6-glucuronide (Oswald et al., 2006b;Oswald et al., 2006a;Zelcer et al., 2005). Therefore, future studies should place greater emphasis on determining the role of drug transporters in pharmacokinetics and pharmacodynamics of drug therapy administered during NAFLD.

Reference List

Abdelmalek M, Ludwig J, and Lindor KD (1995) Two cases from the spectrum of nonalcoholic steatohepatitis. *J.Clin.Gastroenterol.* **20**:127-130.

Abiru S, Migita K, Maeda Y, Daikoku M, Ito M, Ohata K, Nagaoka S, Matsumoto T, Takii Y, Kusumoto K, Nakamura M, Komori A, Yano K, Yatsuhashi H, Eguchi K, and Ishibashi H (2006) Serum cytokine and soluble cytokine receptor levels in patients with non-alcoholic steatohepatitis. *Liver Int.* **26**:39-45.

Bahcecioglu IH, Yalniz M, Ataseven H, Ilhan N, Ozercan IH, Seckin D, and Sahin K (2005) Levels of serum hyaluronic acid, TNF-alpha and IL-8 in patients with nonalcoholic steatohepatitis. *Hepatogastroenterology.* **52**:1549-1553.

Borst P, Evers R, Kool M, and Wijnholds J (2000) A family of drug transporters: the multidrug resistance-associated proteins. *J.Natl.Cancer Inst.* **92**:1295-1302.

Brouwer KL and Jones JA (1990) Altered hepatobiliary disposition of acetaminophen metabolites after phenobarbital pretreatment and renal ligation: evidence for impaired biliary excretion and a diffusional barrier. *J.Pharmacol.Exp.Ther.* **252**:657-664.

Brunt EM and Tiniakos DG (2005) Pathological features of NASH. *Front Biosci.* **10**:1475-1484.

Buchler M, Konig J, Brom M, Kartenbeck J, Spring H, Horie T, and Keppler D (1996) cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats. *J.Biol.Chem.* **271**:15091-15098.

Caldwell SH and Hespenheide EE (2002) Subacute liver failure in obese women. *Am.J.Gastroenterol.* **97**:2058-2062.

Charlton M, Kasparova P, Weston S, Lindor K, Maor-Kendler Y, Wiesner RH, Rosen CB, and Batts KP (2001) Frequency of nonalcoholic steatohepatitis as a cause of advanced liver disease. *Liver Transpl.* **7**:608-614.

Chawla RK, Watson WH, Eastin CE, Lee EY, Schmidt J, and McClain CJ (1998) S-adenosylmethionine deficiency and TNF-alpha in lipopolysaccharide-induced hepatic injury. *Am.J.Physiol.* **275**:G125-G129.

Chen C, Hennig GE, and Manautou JE (2003) Hepatobiliary excretion of acetaminophen glutathione conjugate and its derivatives in transport-deficient (TR-) hyperbilirubinemic rats. *Drug Metab Dispos.* **31**:798-804.

Chen C, Hennig GE, Whiteley HE, Corton JC, and Manautou JE (2000) Peroxisome proliferator-activated receptor alpha-null mice lack resistance to

acetaminophen hepatotoxicity following clofibrate exposure. *Toxicol.Sci.* **57**:338-344.

Chen ZS, Lee K, and Kruh GD (2001) Transport of cyclic nucleotides and estradiol 17-beta-D-glucuronide by multidrug resistance protein 4. Resistance to 6-mercaptopurine and 6-thioguanine. *J.Biol.Chem.* **276**:33747-33754.

Cherrington NJ, Hartley DP, Li N, Johnson DR, and Klaassen CD (2002) Organ distribution of multidrug resistance proteins 1, 2, and 3 (Mrp1, 2, and 3) mRNA and hepatic induction of Mrp3 by constitutive androstane receptor activators in rats. *J.Pharmacol.Exp.Ther.* **300**:97-104.

Cherrington NJ, Slitt AL, Li N, and Klaassen CD (2004) Lipopolysaccharide-mediated regulation of hepatic transporter mRNA levels in rats. *Drug Metab Dispos.* **32**:734-741.

Congiu M, Mashford ML, Slavin JL, and Desmond PV (2002) UDP glucuronosyltransferase mRNA levels in human liver disease. *Drug Metab Dispos.* **30**:129-134.

Donner MG and Keppler D (2001) Up-regulation of basolateral multidrug resistance protein 3 (Mrp3) in cholestatic rat liver. *Hepatology* **34**:351-359.

Farrell GC, Cooksley WG, Hart P, and Powell LW (1978) Drug metabolism in liver disease. Identification of patients with impaired hepatic drug metabolism. *Gastroenterology.* **75**:580-588.

Farrell GC, Cooksley WG, and Powell LW (1979) Drug metabolism in liver disease: activity of hepatic microsomal metabolizing enzymes. *Clin.Pharmacol.Ther.* **26**:483-492.

Figg WD, Dukes GE, Lesesne HR, Carson SW, Songer SS, Pritchard JF, Hermann DJ, Powell JR, and Hak LJ (1995) Comparison of quantitative methods to assess hepatic function: Pugh's classification, indocyanine green, antipyrine, and dextromethorphan. *Pharmacotherapy.* **15**:693-700.

Forrest JA, Finlayson ND, Adjepon-Yamoah KK, and Prescott LF (1977) Antipyrine, paracetamol, and lignocaine elimination in chronic liver disease. *Br.Med.J.* **1**:1384-1387.

Galinsky RE and Levy G (1981) Dose- and time-dependent elimination of acetaminophen in rats: pharmacokinetic implications of cosubstrate depletion. *J.Pharmacol.Exp.Ther.* **219**:14-20.

Geier A, Dietrich CG, Grote T, Beuers U, Prufer T, Fraunberger P, Matern S, Gartung C, Gerbes AL, and Bilzer M (2005) Characterization of organic anion transporter regulation, glutathione metabolism and bile formation in the obese Zucker rat. *J.Hepatol.* **43**:1021-1030.

Geier A, Dietrich CG, Voigt S, Kim SK, Gerloff T, Kullak-Ublick GA, Lorenzen J, Matern S, and Gartung C (2003) Effects of proinflammatory cytokines on rat organic anion transporters during toxic liver injury and cholestasis. *Hepatology* **38**:345-354.

George J, Pera N, Phung N, Leclercq I, Yun HJ, and Farrell G (2003) Lipid peroxidation, stellate cell activation and hepatic fibrogenesis in a rat model of chronic steatohepatitis. *J.Hepatol.* **39**:756-764.

Grafstrom R, Ormstad K, Moldeus P, and Orrenius S (1979) Paracetamol metabolism in the isolated perfused rat liver with further metabolism of a biliary paracetamol conjugate by the small intestine. *Biochem.Pharmacol.* **28**:3573-3579.

Green RM, Beier D, and Gollan JL (1996) Regulation of hepatocyte bile salt transporters by endotoxin and inflammatory cytokines in rodents. *Gastroenterology* **111**:193-198.

Gregus Z, Madhu C, and Klaassen CD (1988) Species variation in toxication and detoxication of acetaminophen in vivo: a comparative study of biliary and urinary excretion of acetaminophen metabolites. *J.Pharmacol.Exp.Ther.* **244**:91-99.

Gregus Z, Madhu C, and Klaassen CD (1990) Effect of microsomal enzyme inducers on biliary and urinary excretion of acetaminophen metabolites in rats. Decreased hepatobiliary and increased hepatovascular transport of acetaminophen-glucuronide after microsomal enzyme induction. *Drug Metab.Dispos.* **18**:10-19.

Howie D, Adriaenssens PI, and Prescott LF (1977) Paracetamol metabolism following overdosage: application of high performance liquid chromatography. *J.Pharm.Pharmacol.* **29**:235-237.

Jonker JW, Buitelaar M, Wagenaar E, van der Valk MA, Scheffer GL, Scheper RJ, Plosch T, Kuipers F, Elferink RP, Rosing H, Beijnen JH, and Schinkel AH (2002) The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. *Proc.Natl.Acad.Sci.U.S.A.* **99**:15649-15654.

Keppler D, Kamisako T, Leier I, Cui Y, Nies AT, Tsujii H, and Konig J (2000) Localization, substrate specificity, and drug resistance conferred by conjugate export pumps of the MRP family. *Adv.Enzyme Regul.* **40**:339-349.

Keppler D and Konig J (1997) Expression and localization of the conjugate export pump encoded by the MRP2 (cMRP/cMOAT) gene in liver. *FASEB Journal.* **11**:509-516.

Keppler D and Konig J (2000) Hepatic secretion of conjugated drugs and endogenous substances. *Semin.Liver Dis.* **20**:265-272.

Keppler D, Leier I, and Jedlitschky G (1997) Transport of glutathione conjugates and glucuronides by the multidrug resistance proteins MRP1 and MRP2. *Biol.Chem.* **378**:787-791.

Konig J, Rost D, Cui Y, and Keppler D (1999) Characterization of the human multidrug resistance protein isoform MRP3 localized to the basolateral hepatocyte membrane. *Hepatology* **29**:1156-1163.

Kopelman PG (2000) Obesity as a medical problem. *Nature* **404**:635-643.

Kubitz R, Wettstein M, Warskulat U, and Haussinger D (1999) Regulation of the multidrug resistance protein 2 in the rat liver by lipopolysaccharide and dexamethasone [see comments]. *Gastroenterology* **116**:401-410.

Kugelmas M, Hill DB, Vivian B, Marsano L, and McClain CJ (2003) Cytokines and NASH: a pilot study of the effects of lifestyle modification and vitamin E. *Hepatology* **38**:413-419.

Kuroda M, Kobayashi Y, Tanaka Y, Itani T, Mifuji R, Araki J, Kaito M, and Adachi Y (2004) Increased hepatic and renal expressions of multidrug resistance-associated protein 3 in Eisai hyperbilirubinuria rats. *J.Gastroenterol.Hepatol.* **19**:146-153.

Levy G and Yamada H (1971) Drug biotransformation interactions in man. 3. Acetaminophen and salicylamide. *J.Pharm.Sci.* **60**:215-221.

Liu RH, Mizuta M, Kurose T, and Matsukura S (2002) Early events involved in the development of insulin resistance in Zucker fatty rat. *Int.J.Obes.Relat Metab Disord.* **26**:318-326.

Lohr JW, Willsky GR, and Acara MA (1998) Renal drug metabolism. *Pharmacol.Rev.* **50**:107-141.

Manautou JE, de Waart DR, Kunne C, Zelcer N, Goedken M, Borst P, and Elferink RO (2005) Altered disposition of acetaminophen in mice with a disruption of the Mrp3 gene. *Hepatology.* **42**:1091-1098.

McCullough AJ (2006) Pathophysiology of nonalcoholic steatohepatitis. *J.Clin.Gastroenterol.* **40**:S17-S29.

Mizuno N, Suzuki M, Kusuhara H, Suzuki H, Takeuchi K, Niwa T, Jonker JW, and Sugiyama Y (2004) Impaired renal excretion of 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole (E3040) sulfate in breast cancer resistance protein (BCRP1/ABCG2) knockout mice. *Drug Metab Dispos.* **32**:898-901.

Moldeus P (1978) Paracetamol metabolism and toxicity in isolated hepatocytes from rat and mouse. *Biochem.Pharmacol.* **27**:2859-2863.

Nadai M, Zhao YL, Wang L, Nishio Y, Takagi K, Kitaichi K, Takagi K, Yoshizumi H, and Hasegawa T (2001) Endotoxin impairs biliary transport of sparfloxacin and its glucuronide in rats. *Eur.J.Pharmacol.* **432**:99-105.

Nakanishi T, Doyle LA, Hassel B, Wei Y, Bauer KS, Wu S, Pumplin DW, Fang HB, and Ross DD (2003) Functional characterization of human breast cancer resistance protein (BCRP, ABCG2) expressed in the oocytes of Xenopus laevis. *Mol.Pharmacol.* **64**:1452-1462.

Narang AP, Datta DV, and Mathur VS (1985) Impairment of drug elimination in patients with liver disease. *Int.J.Clin.Pharmacol.Ther.Toxicol.* **23**:28-32.

Narang AP, Datta DV, Nath N, and Mathur VS (1981) Pharmacokinetic study of chloramphenicol in patients with liver disease. *Eur.J.Clin.Pharmacol.* **20**:479-483.

Narang AP, Datta DV, Nath N, and Mathur VS (1982) Impairment of hepatic drug metabolism in patients with acute viral hepatitis. *Eur.J.Drug Metab Pharmacokinet.* **7**:255-258.

Oswald S, Haenisch S, Fricke C, Sudhop T, Remmler C, Giessmann T, Jedlitschky G, Adam U, Dazert E, Warzok R, Wacke W, Cascorbi I, Kroemer HK, Weitschies W, von Bergmann K, and Siegmund W (2006a) Intestinal expression of P-glycoprotein (ABCB1), multidrug resistance associated protein 2 (ABCC2), and uridine diphosphate-glucuronosyltransferase 1A1 predicts the disposition and modulates the effects of the cholesterol absorption inhibitor ezetimibe in humans. *Clin.Pharmacol.Ther.* **79**:206-217.

Oswald S, Westrup S, Grube M, Kroemer HK, Weitschies W, and Siegmund W (2006b) Disposition and sterol-lowering effect of ezetimibe in multidrug resistance-associated protein 2-deficient rats. *J.Pharmacol.Exp.Ther.* **318**:1293-1299.

Pizarro M, Balasubramaniyan N, Solis N, Solar A, Duarte I, Miquel JF, Suchy FJ, Trauner M, Accatino L, Ananthanarayanan M, and Arrese M (2004) Bile secretory function in the obese Zucker rat: evidence of cholestasis and altered canalicular transport function. *Gut* **53**:1837-1843.

Powell EE, Cooksley WG, Hanson R, Searle J, Halliday JW, and Powell LW (1990) The natural history of nonalcoholic steatohepatitis: a follow-up study of forty-two patients for up to 21 years. *Hepatology* **11**:74-80.

Schaub TP, Kartenbeck J, Konig J, Vogel O, Witzgall R, Kriz W, and Keppler D (1997) Expression of the conjugate export pump encoded by the mrp2 gene in the apical membrane of kidney proximal tubules. *J.Am.Soc.Nephrol.* **8**:1213-1221.

Slattery JT and Levy G (1979) Pharmacokinetic model of acetaminophen elimination. *Am.J.Hosp.Pharm.* **36**:440.

Slitt AL, Cherrington NJ, Maher JM, and Klaassen CD (2003) Induction of multidrug resistance protein 3 in rat liver is associated with altered vectorial excretion of acetaminophen metabolites. *Drug Metab Dispos.* **31**:1176-1186.

Soroka CJ, Lee j, Azzaroli F, and Boyer JL (2001) Cellular localization and up-regulation of multidrug resistance-associated protein 3 in hepatocytes and cholangiocytes during obstructive cholestasis in rat liver. *Hepatology* **33**:783-791.

Starkel P, Sempoux C, Leclercq I, Herin M, Deby C, Desager JP, and Horsmans Y (2003) Oxidative stress, KLF6 and transforming growth factor-beta up-regulation differentiate non-alcoholic steatohepatitis progressing to fibrosis from uncomplicated steatosis in rats. *J.Hepatol.* **39**:538-546.

Tanaka Y, Slitt AL, Leazer TM, Maher JM, and Klaassen CD (2005) Tissue distribution and hormonal regulation of the breast cancer resistance protein (Bcrp/Abcg2) in rats and mice. *Biochem.Biophys.Res.Commun.* **326**:181-187.

Vendemiale G, Grattagliano I, Altomare E, Turturro N, and Guerrieri F (1996) Effect of acetaminophen administration on hepatic glutathione compartmentation and mitochondrial energy metabolism in the rat. *Biochem.Pharmacol.* **52**:1147-1154.

Westphal JF and Brogard JM (1997) Drug administration in chronic liver disease. *Drug Saf.* **17**:47-73.

Whiting JF, Green RM, Rosenbluth AB, and Gollan JL (1995) Tumor necrosis factor-alpha decreases hepatocyte bile salt uptake and mediates endotoxin-induced cholestasis. *Hepatology* **22**:1273-1278.

Xiong H, Suzuki H, Sugiyama Y, Meier PJ, Pollack GM, and Brouwer KL (2002) Mechanisms of impaired biliary excretion of acetaminophen glucuronide after acute phenobarbital treatment or phenobarbital pretreatment. *Drug Metab Dispos.* **30**:962-969.

Xiong H, Turner KC, Ward ES, Jansen PL, and Brouwer KL (2000) Altered hepatobiliary disposition of acetaminophen glucuronide in isolated perfused livers from multidrug resistance-associated protein 2-deficient TR(-) rats [In Process Citation]. *J.Pharmacol.Exp.Ther.* **295**:512-518.

Zamek-Gliszczynski MJ, Hoffmaster KA, Humphreys JE, Tian X, Nezasa K, and Brouwer KL (2006) Differential involvement of Mrp2 (Abcc2) and Bcrp (Abcg2) in biliary excretion of 4-methylumbelliferyl glucuronide and sulfate in the rat. *J.Pharmacol.Exp.Ther.* **319**:459-467.

Zamek-Gliszczynski MJ, Hoffmaster KA, Tian X, Zhao R, Polli JW, Humphreys JE, Webster LO, Bridges AS, Kalvass JC, and Brouwer KL (2005) Multiple mechanisms are involved in the biliary excretion of acetaminophen sulfate in the rat: role of Mrp2 and Bcrp1. *Drug Metab Dispos.* **33**:1158-1165.

Zamek-Gliszczynski MJ, Nezasa K, Tian X, Bridges AS, Lee K, Belinsky MG, Kruh GD, and Brouwer KL (2006) Evaluation of the role of multidrug resistance-associated protein (mrp) 3 and mrp4 in hepatic basolateral excretion of sulfate and glucuronide metabolites of acetaminophen, 4-methylumbelliferone, and harmol in abcc3-/- and abcc4-/- mice. *J.Pharmacol.Exp.Ther.* **319**:1485-1491.

Zelcer N, van de WK, Hillebrand M, Sarton E, Kuil A, Wielinga PR, Tephly T, Dahan A, Beijnen JH, and Borst P (2005) Mice lacking multidrug resistance protein 3 show altered morphine pharmacokinetics and morphine-6-glucuronide antinociception. *Proc.Natl.Acad.Sci.U.S.A.* **102**:7274-7279.

DMD Fast Forward. Published on July 19, 2007 as DOI: 10.1124/dmd.107.015107
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURES

Figure 1. H & E- and trichrome-stained sections of rat liver following 8 weeks of control, high-fat or MCD diet. Following 8 weeks on a control, high-fat or MCD diet, 5-μm liver cryosections from each group were stained with hematoxylin and eosin (A-C) or Masson's trichrome (D-F). Histologic analyses were conducted using light microscopy at 40x magnification.

Figure 2. Efflux transporter mRNA levels in the liver following 8 weeks of feeding on the respective high-fat and MCD diets. Following 8 weeks on a control, high-fat or MCD diet, total RNA was isolated from the liver of male Sprague-Dawley rats. Levels of mRNA were analyzed by the bDNA assay and expressed as relative light units (RLU) ± (standard error of the mean) SEM (n = 5). Asterisks (*) indicate a significant difference from the control group ($p \leq 0.05$).

Figure 3. Efflux transporter protein levels in liver following 8 weeks of feeding on the respective high-fat and MCD diets. Following 8 weeks on a control, high-fat or MCD diet, liver crude membrane fractions from male Sprague-Dawley rats were analyzed by Western blot (40 μg of protein/lane). Transporter protein bands were quantified using ImageJ software and are expressed as percent of the control group ± SEM (n = 5). Asterisks (*) indicate a significant difference from the control group ($p \leq 0.05$).

Figure 4. Effect of high-fat and MCD diets on bile flow. Following 8 weeks on a control, high-fat or MCD diet, APAP excretion experiments were conducted. The left carotid artery and bile duct were cannulated and APAP was administered. Immediately after the APAP dose, bile was collected in 15 minute segments over 90 minutes. The data are represented as the mean ± SEM. Asterisks (*) indicate a significant difference from the control group ($p \leq 0.05$).

Figure 5. Effect of high-fat and MCD diets on biliary excretion of APAP and APAP metabolites. Experimental conditions were the same as for Figure 6. The data are represented as the mean concentration ± SEM. Asterisks (*) indicate a significant difference from the control group ($p \leq 0.05$).

Figure 6. Effect of high-fat and MCD diets on APAP and APAP-GLUC metabolite concentration in liver. Liver samples were collected at the end of the APAP excretion experiment and stored at -80°C before analysis by HPLC as described under Materials and Methods. The APAP-GSH, APAP-SULF and APAP-CG/CYS metabolites were not detectable in livers from all three groups of rats. The data are represented as the mean concentration ± SEM. Asterisks (*) indicate a significant difference from the control group ($p \leq 0.05$).

Figure 7. Effect of high-fat and MCD diets on plasma concentration of APAP and APAP metabolites. Experimental conditions were the same as for Figure 6. The data are represented as the mean concentration ± SEM. The

APAP-NAC and APAP-CG/CYS metabolites were not detectable in the plasma of all three groups of rats. The data are represented as the mean concentration ± SEM. Asterisks (*) indicate a significant difference from the control group ($p \leq 0.05$).

Figure 8. Effect of high-fat and MCD diets on urinary excretion of APAP and APAP metabolites. Experimental conditions were the same as for Figure 6. The data are represented as the mean concentration ± SEM. This figure shows the cumulative concentration of APAP and its respective metabolites in the urine 90 minutes after APAP administration. The data are represented as the mean concentration ± SEM. Asterisks (*) indicate a significant difference from the control group ($p \leq 0.05$).

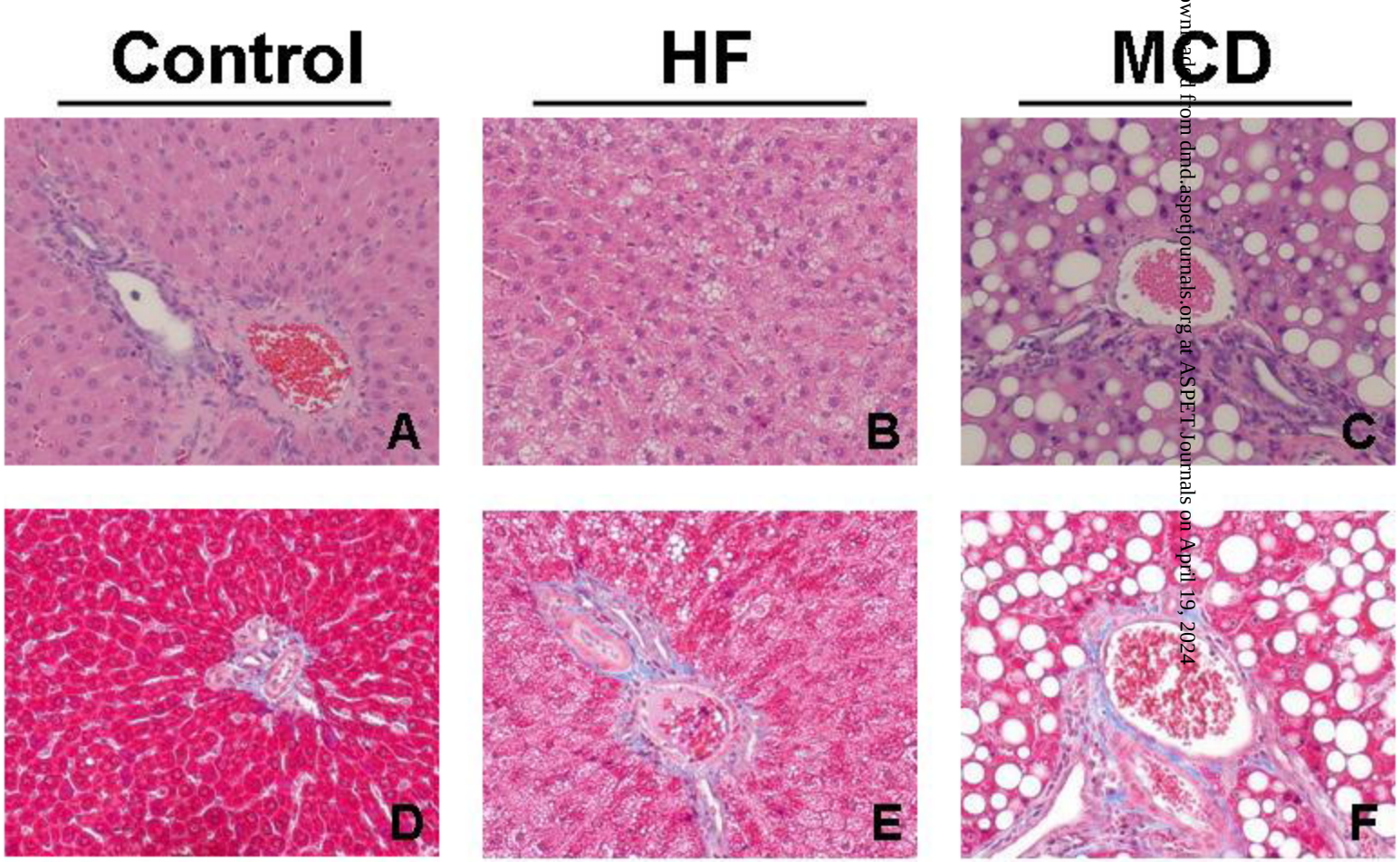

Figure 1.

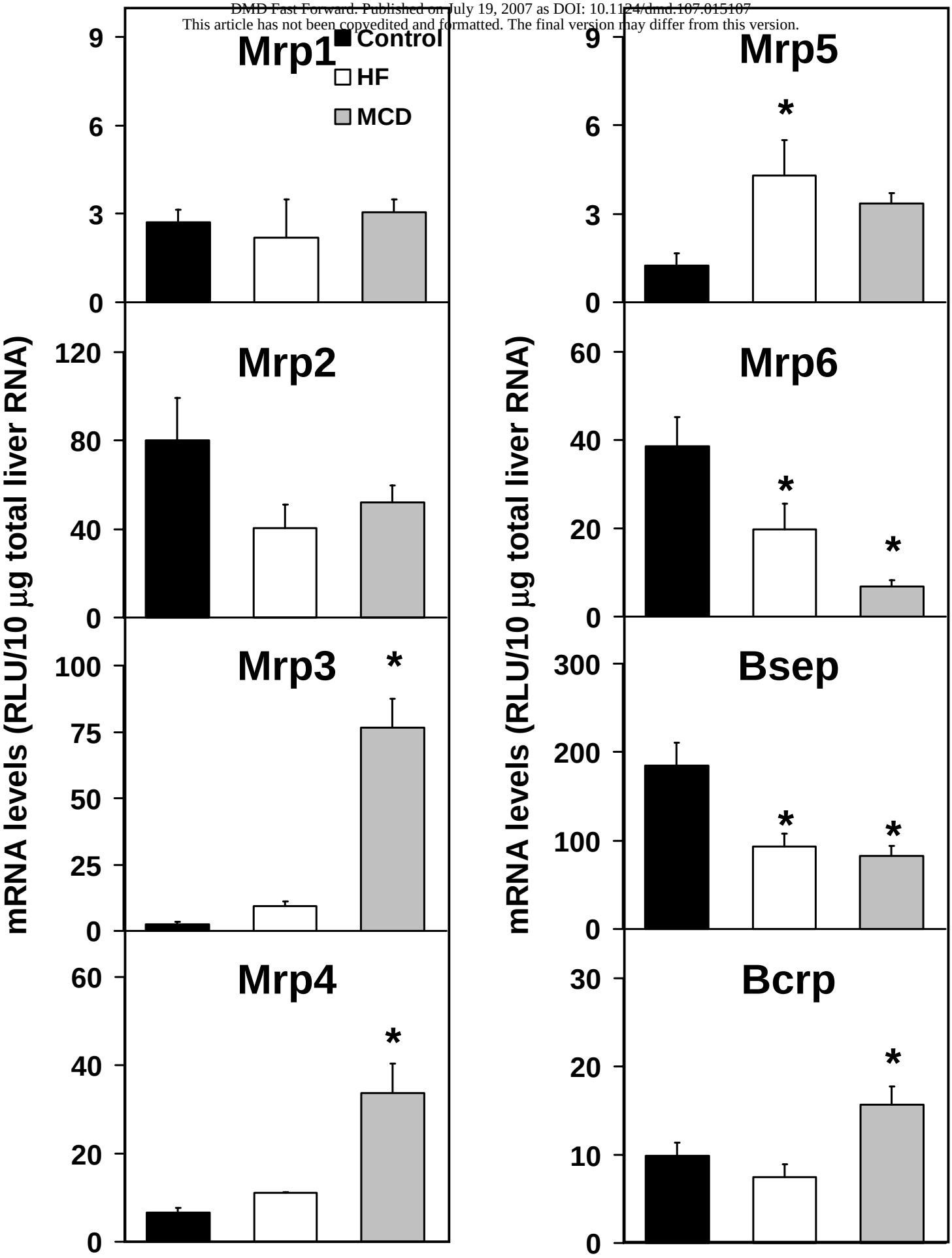

Figure 2.

DMD Fast Forward. Published on July 19, 2007 as DOI: 10.1124/dmd.107.015107
This article has not been copyedited and formatted. The final version may differ from this version.

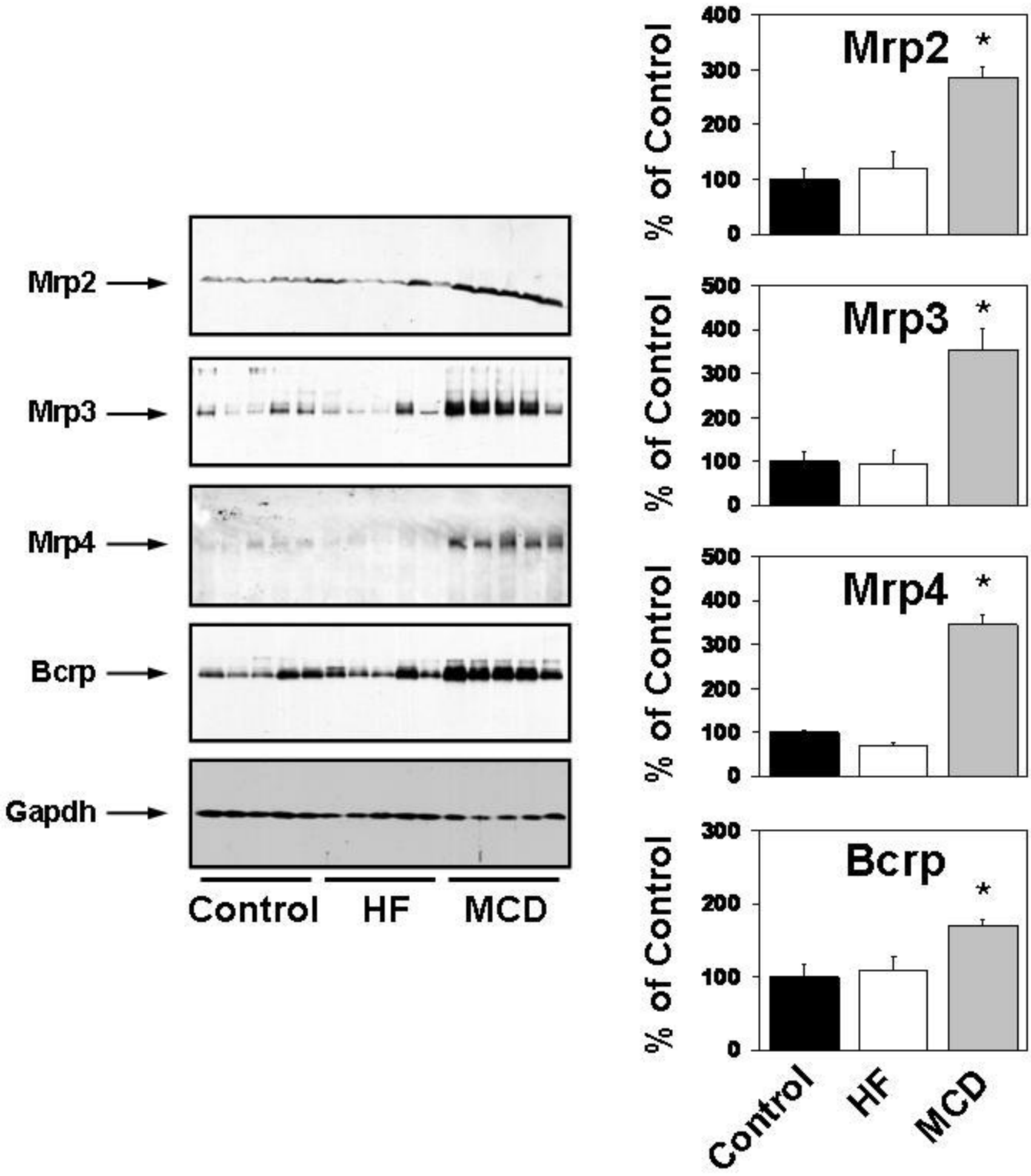

Figure 3.

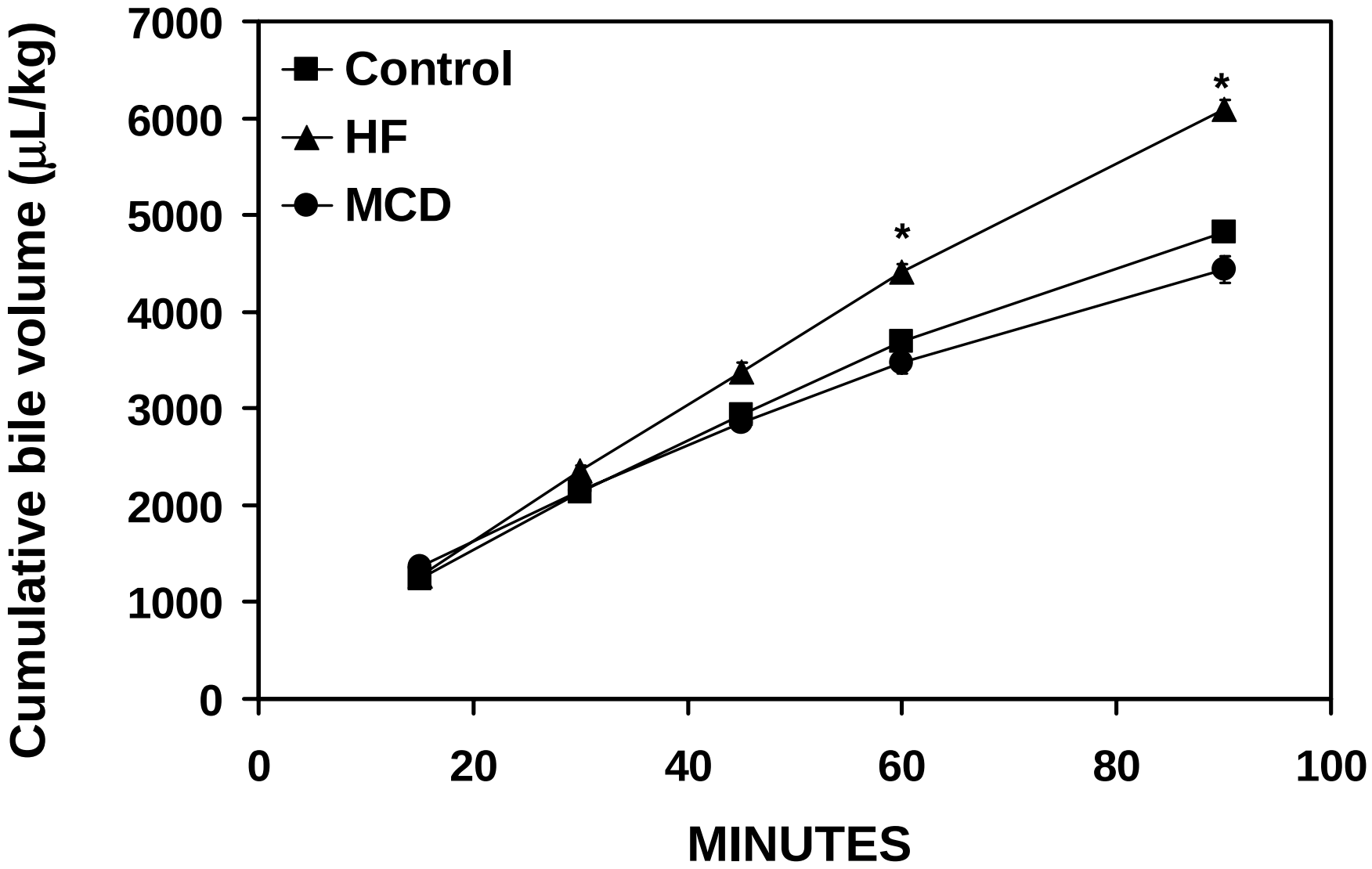

Figure 4.

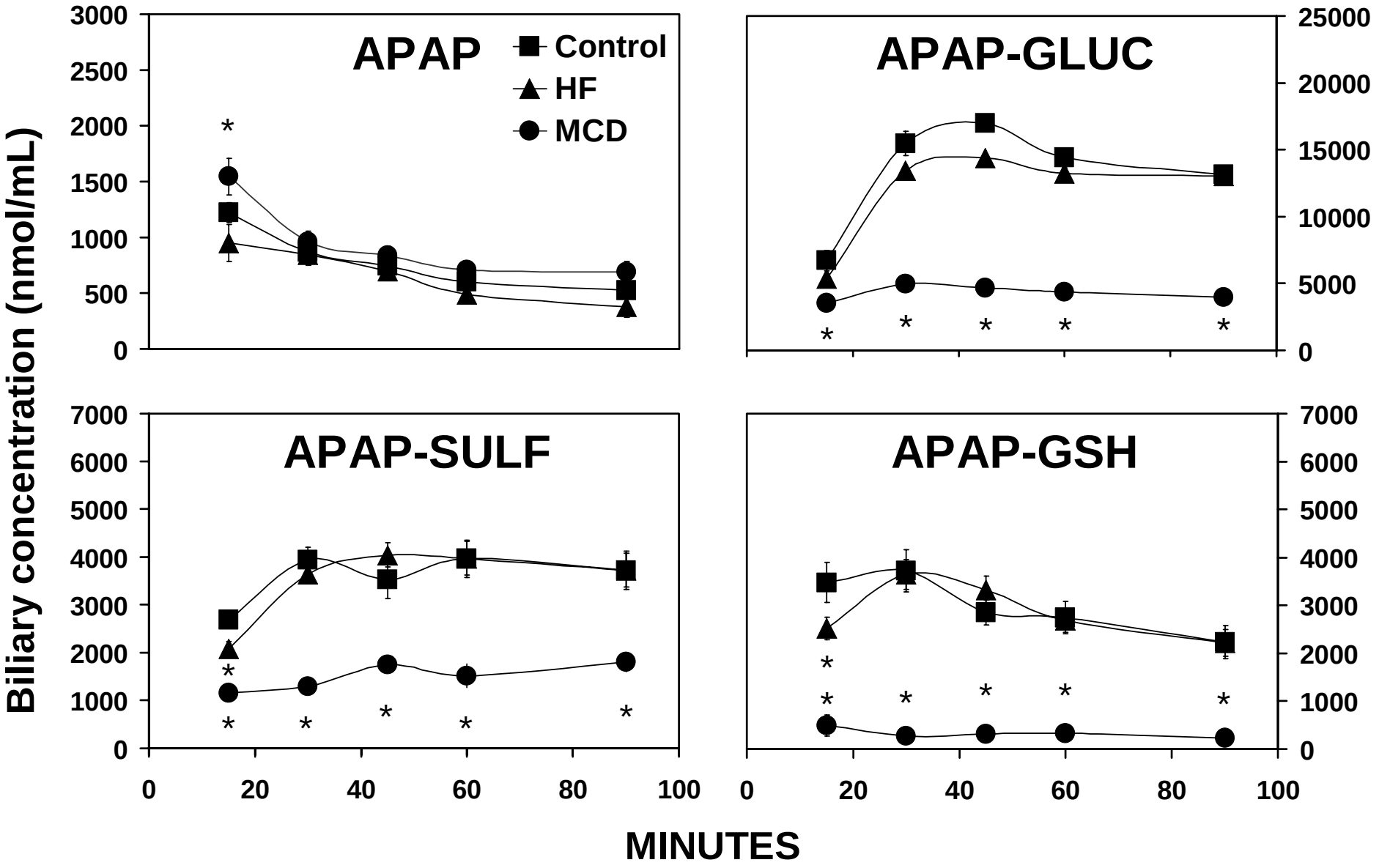

Figure 5.

DMD Fast Forward. Published on July 19, 2007 as DOI: 10.1124/dmd.107.015107
This article has not been copyedited and formatted. The final version may differ from this version.

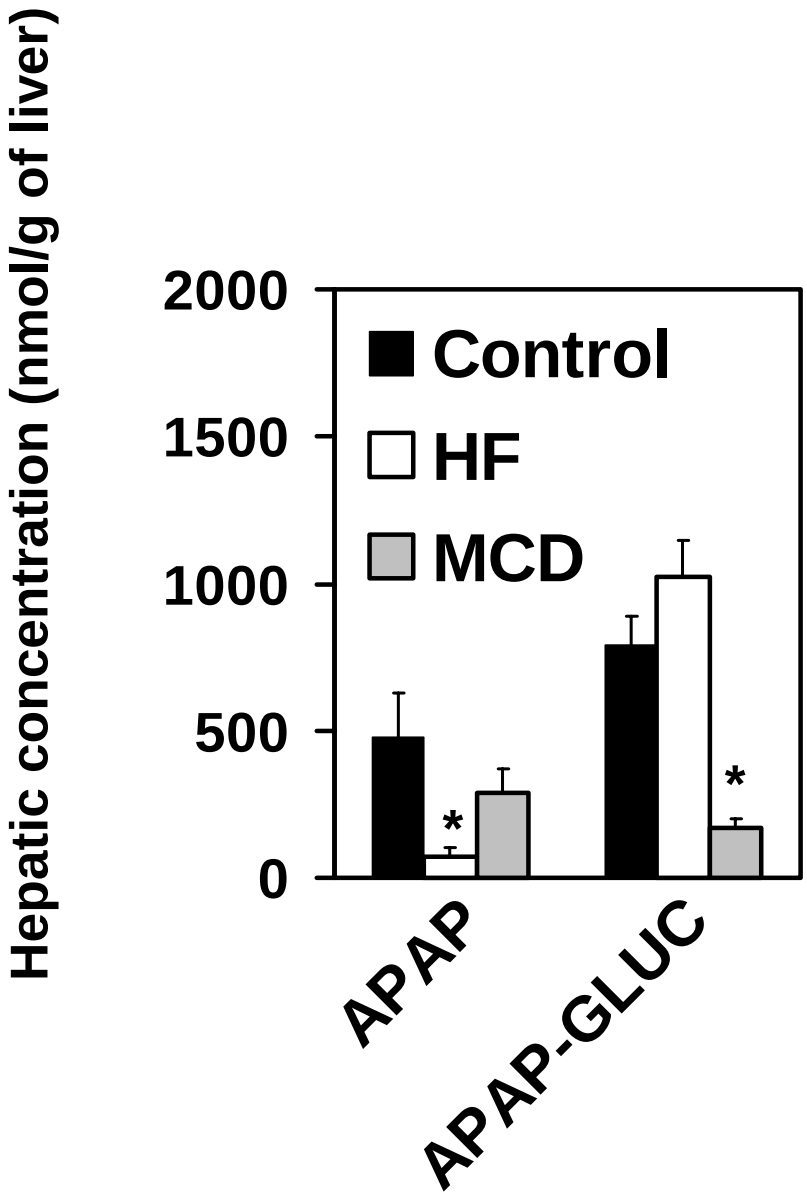

Figure 6.

DMD Fast Forward. Published on July 19, 2007 as DOI: 10.1124/dmd.107.015107
This article has not been copyedited and formatted. The final version may differ from this version.

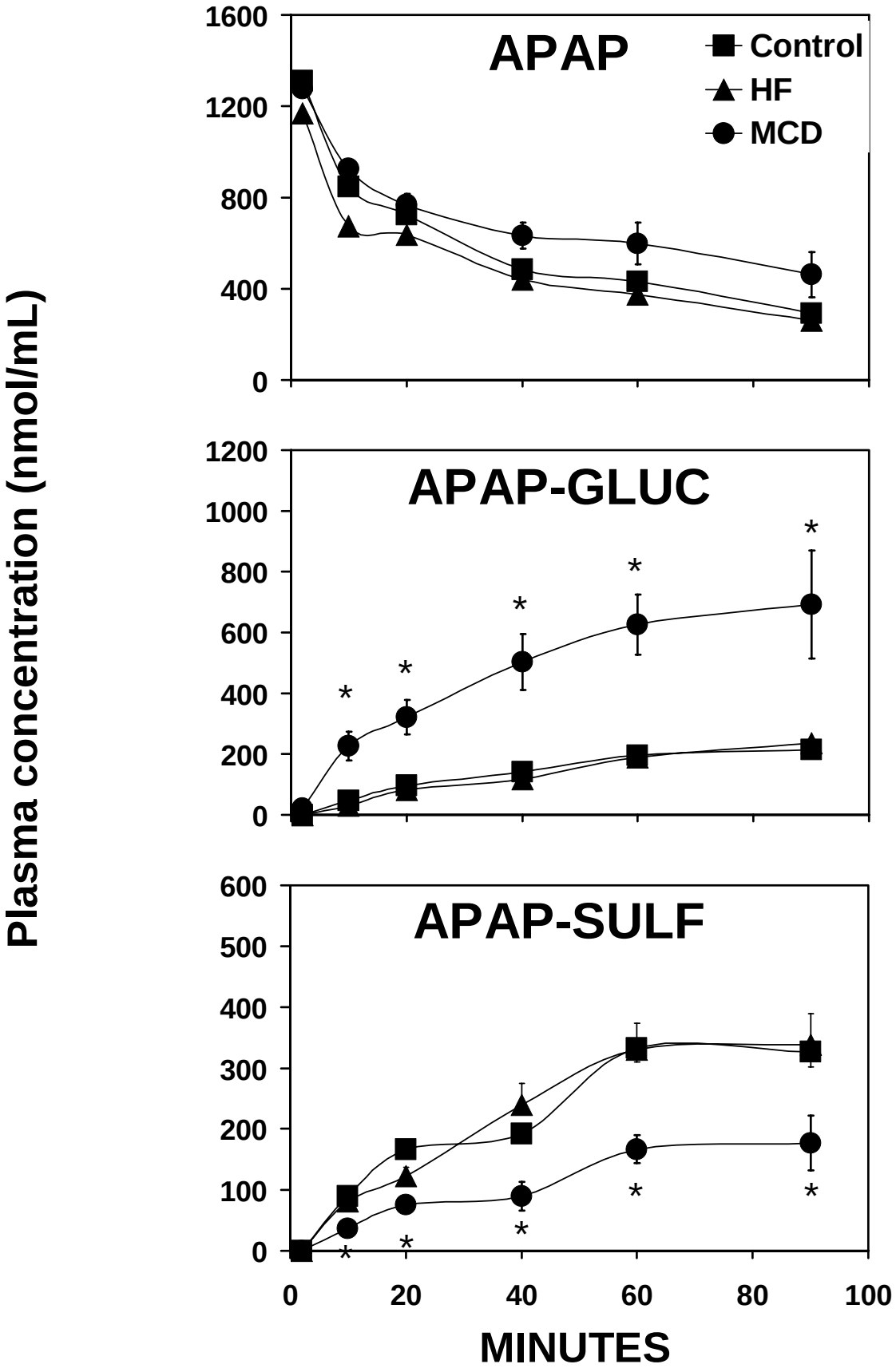

Figure 7.

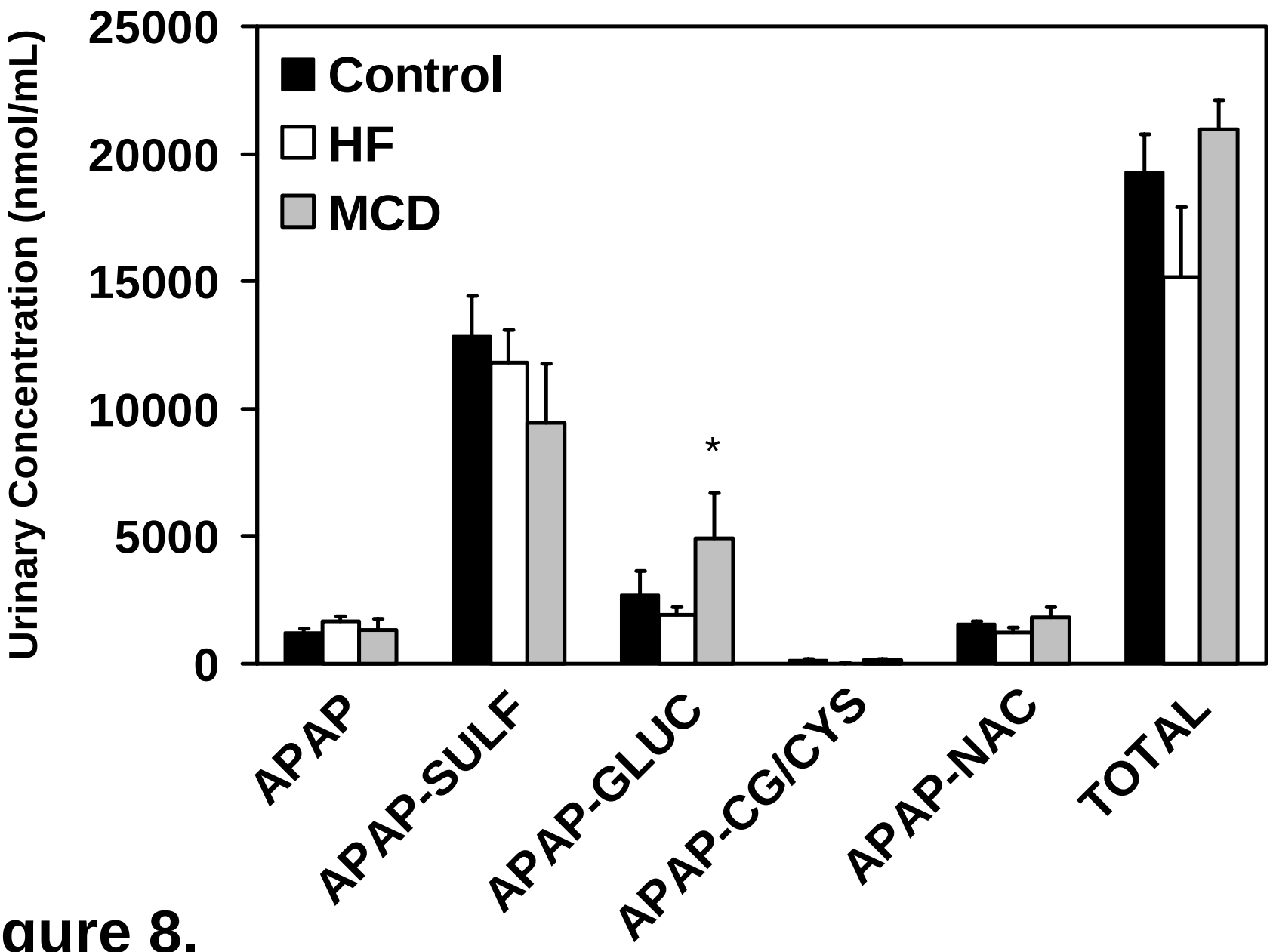

Figure 8.