

**UDP-GLUCURONOSYLTRANSFERASE EXPRESSION IN MOUSE
LIVER IS INCREASED IN OBESITY AND FASTING-INDUCED
STEATOSIS**

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Running title: Obesity and fasting induce UGT expression

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Non-standard Abbreviations: Abcc, ATP-binding cassette, sub-family C; AhR, aryl hydrocarbon receptor; cAMP, cyclic adenosine 3',5'-monophosphate; CAR, constitutive androstane receptor; FFA, free fatty acid; Gclc, glutamate-cysteine ligase, catalytic subunit; Nqo1, NAD(P)H dehydrogenase, quinone 1; Nrf2, nuclear factor (erythroid-derived 2)-like 2; PGC1 α , peroxisome proliferator-activated receptor- γ coactivator-1 α ; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; RXR, retinoid X receptor; TG, triglyceride; UGT, UDP-glucuronosyltransferase; UDP-GA, UDP-glucuronic acid.

Abstract:

UDP-glucuronosyltransferases (Ugt) catalyze phase-II conjugation reactions with glucuronic acid, which enhances chemical polarity and the elimination from the body. Few studies address whether Ugt expression and activity are affected by liver disease, such as steatosis. The purpose of this study was to determine whether steatosis induced by obesity or fasting could affect liver Ugt mRNA expression and activity. Male C57BL/6J and *Lep*^{ob/ob} (ob/ob) mice were fed *ad libitum* or food withheld for 24 hours. In steatotic livers of ob/ob mice, Ugt1a1, 1a6, 1a9, 2a3, 3a1, and 3a2 mRNA expression increased. Fasting, which also induced steatosis, increased hepatic Ugt1a1, 1a6, 1a7, 1a9, 2b1, 2b5, 2a3, 3a1, and 3a2 mRNA expression in mouse liver. Correspondingly, acetaminophen glucuronidation increased by 47% in hepatic microsomes from ob/ob mice compared to C57BL/6J mice, but not after fasting. In both steatosis models, Ugt induction was accompanied by increased AhR, CAR, PPAR α , PXR, Nrf2, and PGC1 α mRNA expression. Additionally, fasting increased CAR, PPAR, and Nrf2 binding activity. The work points to hepatic triglyceride concentrations corresponding with nuclear receptor and Ugt expression. The findings indicate that steatosis significantly alters hepatic Ugt expression and activity, which could have a significant impact on determining circulating hormone levels, drug efficacy, and environmental chemical clearance.

Introduction:

UDP-glucuronosyltransferases are a family of biotransformation enzymes that catalyze Phase-II biotransformation reactions, conjugate lipophilic substrates with glucuronic acid, and aid in transporter-mediated excretion into bile and urine. Glucuronidation is a major detoxification pathway for both endogenous and exogenous compounds, and is becoming increasingly important for clearance and elimination of the top 200 drugs (Williams et al., 2004). Endogenous Ugt substrates comprise numerous steroids and metabolic products, including bilirubin, steroidal hormones, thyroid hormones, biliary acids, hyodeoxycholic acid, and vitamins. Numerous xenobiotics, including acetaminophen, morphine, propofol, chloramphenicol, and nonsteroidal anti-inflammatory drugs, as well as environmental compounds such as Bisphenol A (Hanioka et al., 2008) are glucuronidated by UDP-glucuronosyltransferase.

Based on the amino acid sequence similarity, the Ugt superfamily is divided into Ugt1 and Ugt2. The Ugt1 gene consists of a unique first exon that encodes the N-terminal domain, and splices with common exons 2 to 5 that create the common C-terminal domain. Each Ugt1a member contains gene-specific promoter regions (Mackenzie et al., 1997). In mice, the Ugt1a gene contains 14 first exons, coding nine enzymes (Ugt1a1, 1a2, 1a5, 1a6a, 1a6b, 1a7c, 1a8, 1a9, and 1a10) and five pseudogenes (Ugt1a3, 1a4, 1a7a, 1a7b, and 1a11) (Zhang et al., 2004), mediate drug, hormone, and bilirubin glucuronidation. The Ugt2 isoforms are encoded by separate genes composed of six individual exons, further subdivided into Ugt2a and Ugt2b subfamilies. There are three Ugt2a gene duplication products, Ugt2a1-a3. The seven Ugt2b genes in mice include Ugt2b1, 2b5, 2b34-2b38. Additionally, a novel Ugt3a family has been

recently identified in humans and rodents, giving rise to two individual gene products in mice, Ugt3a1 and 3a2, located on mouse chromosome 15 (Mackenzie et al., 2005).

It is well described that Ugt induction in liver occurs with microsomal enzyme inducer treatment and this induction is mediated through nuclear receptor- and transcription factor-dependent mechanisms (Shelby and Klaassen, 2006; Buckley and Klaassen, 2009). Peroxisome proliferator-activated receptor alpha (PPAR α), pregnane-X-receptor (PXR), constitutive androstane receptor (CAR), nuclear factor (erythroid-derived 2)-like 2 (Nrf2), and aryl hydrocarbon receptor (AhR) have been implicated in the regulation of Ugt expression. PPAR-response elements are present in the human UGT2B4 promoter and PPAR α :RXR α binding has been described to drive the gene expression (Barbier et al., 2003). Pregnenolone-16-carbonitrile, a PXR ligand, upregulated Ugt1a1, 1a4, and 1a6 mRNA and protein levels in livers and intestines of mice in a PXR-dependent manner (Chen et al., 2005). Phenobarbital increased UGT1A1 gene transcription by CAR-dependent mechanism (Sugatani et al., 2005). Tetrachlorodibenzo-p-dioxin (TCDD), a potent AhR activator, induced Ugt1a6, 1a9, 2b34, 2b35, 2b36 mRNA expression in liver, which was Nrf2-dependent (Yeager et al., 2009). Human UGT1A1 expression was markedly increased in intestines and livers of transgenic mice treated with tert-butylhydroquinone, suggesting Nrf2-Keap1 signaling involvement in UGT1A1 induction (Yueh and Tukey, 2007).

Obesity has been cited as a major health hazard factor in the United States for recent years. A common consequence of obesity and diabetes is the development of hepatic steatosis or “fatty liver”, which is the accumulation of triglyceride in hepatocytes. In the United States, it is estimated that one third of the general population has non-alcoholic liver disease (NAFLD), which is associated with obesity and consumption of a diet rich in fat and carbohydrates (Sanyal,

2011). Moreover, type-2 diabetes increases the risk of steatosis. Conversely, steatosis can also be produced by food deprivation, as fat is mobilized from adipose tissue to liver for acetyl CoA production. Steatosis has been observed with anorexia (Sakada et al., 2006). Few studies have addressed how steatosis can impact Ugt expression and activity, and whether excess fat stores can counter fasting-mediated alterations in enzyme expression or activity.

With obesity and obesity-associated hepatic steatosis increasing, the need to understand how Ugt expression is affected under fed and nutrient-deprived conditions to better predict adverse drug reactions, drug-induced liver injury, and alterations in drug pharmacokinetics. The current study aimed to determine whether Ugt expression is altered in livers of obese (ob/ob) mice, and how obese mice regulate Ugt expression after food deprivation. Our results demonstrate Ugt expression increases correspondingly with increased hepatic triglyceride content and nuclear receptor expression, and that obesity conferred some resistance to fasting-induced loss of Ugt activity.

Materials and Methods:

Supplies and reagents. 8-Bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP), acetic acid, acetaminophen, acetaminophen glucuronide, polyethylene glycol hexadecyl ether (Brij-58), ethylenediaminetetraacetic acid (EDTA), magnesium chloride (MgCl₂), paraxanthine (1,7-dimethylxanthine), perchloric acid, potassium chloride, sodium pyrophosphate, sucrose, trizma hydrochloride, UDP-glucuronic acid (UDP-GA), and protein protease inhibitor were purchased from Sigma (St. Louis, MO). Ethanol, methanol, chloroform, Triton X-100 were obtained from Fisher Scientific (Fair Lawn, NJ). All utilized chemicals were of the highest purity available for analytical research.

Animal care. Male eight-week-old C57BL/6J and Lep^{ob/ob} mice were purchased from Jackson Laboratories (Bar Harbor, ME) and acclimated for a minimum of one week in a temperature/humidity-controlled facility. Mice were fed Teklad Rodent Diet 7012 (Harlan, Madison, WI) and allowed access to water *ad libitum* unless otherwise noted. All procedures were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Rhode Island Animal Care and Use Committee.

Fasting studies. Mice were divided into four groups: C57BL/6J fed *ad libitum* (C57-Fed), C57BL/6J food withheld (C57-Fasted), Lep^{ob/ob} fed *ad libitum* (ob/ob-Fed), and Lep^{ob/ob} food withheld (ob/ob-Fasted), n=8 for each group. The day before fasting, mice were housed singly and habituated for one day. Mice were allowed access to water and food *ad libitum* or water only. Blood and liver tissue were collected 24 hours after food was withheld. Blood was centrifuged at 5,000 x g for 15 minutes at 4 °C; serum was isolated and stored at -80 °C. Two portions of the left lobe of each liver were fixed in 10% formalin. The remainder of the liver was snap frozen in liquid nitrogen and stored at -80 °C.

Hematoxylin and eosin staining of liver sections. Liver tissue was fixed in buffered formalin for 24 hours, transferred to 70% ethanol, and then processed for paraffin embedding. Paraffin sections (5 μm) were cut and stained with hematoxylin and eosin (H&E). All procedures were performed according to typical histology protocols as performed by AML Laboratories (Baltimore, MD).

Glucose assay. Serum glucose levels were analyzed using a spectrophotometric glucose assay kit (Cayman Chemical Company, MI) according to the manufacturer's protocol. Five hundred μL of glucose assay reagent was added to 5 μL of serum or standard and incubated for 10 minutes at 37 $^{\circ}\text{C}$. The absorbance was measured at 500-520 nm by using a 96-well plate reader.

Serum cytokine and hormone quantification. Serum cytokine concentrations were determined using a Milliplex MAP kit (Millipore, MA) according to the manufacturer's protocol. Ten μL serum was added and incubated with specific antibody-immobilized beads with agitation on a plate shaker overnight at 4 $^{\circ}\text{C}$. The plate was then washed with 200 μL wash buffer/well three times. Fifty μL of detection antibodies was added to each well and incubated for 30 minutes with agitation. Further 50 μL streptavidin-phycoerythrin was added to the well and incubated for another 30 minutes. The plate was washed and 100 μL sheath fluid was added. The median fluorescent intensity was measured on Luminex Bio-plexTM 200 and data was acquired with Bio-plex Manager 5.0 software (Bio-Rad, CA).

Primary mouse hepatocyte culture, RNA isolation, and quantitative real-time PCR.

Primary mouse hepatocytes were isolated from 2-3 month-old male C57BL/6J or ob/ob mouse livers using a two-step collagenase perfusion; 1×10^6 cells/well in 2 mL completed medium (MEM supplied with 10% FBS) were seeded on collagen-coated 6-well plates. After cell attachment (~4 h), they were cultured in serum-free MEM containing 1% ITS supplement

(Invitrogen, CA). Around 24 hours post-plating, hepatocytes were treated with vehicle or 0.1 mM 8-Br-cAMP for another 24 hours. Total RNA was isolated from cells or 50 mg tissue using TRIzol reagent (Invitrogen, CA) according to the manufacturer's instructions. One microgram of total RNA was converted to single-stranded cDNA using oligo(dT)₁₈ primers and mRNA levels were quantified by quantitative real-time PCR (QPCR) using Roche Lightcycler detection system (Roche Applied Science, Mannheim, Germany). Samples were run by using SYBR green and compared with levels of 18S rRNA as a reference housekeeping gene. QPCR conditions were optimized for each gene using appropriate forward and reverse primers. The primers used are listed in supplemental Table S1. Multiple housekeeping genes were screened (e.g. GAPDH and β -actin), but 18S rRNA was selected because it showed the least change with steatosis. All oligonucleotides were synthesized by Invitrogen Inc., CA.

Quantification of serum and hepatic lipid concentrations. Liver tissues (50 mg) were homogenized at 4 °C in 1 mL PBS, 200 μ L aliquot of the homogenates were used and the lipids were extracted with 3.75 mL of chloroform-methanol (2:1; v/v). The lipid residue was resuspended in 200 μ L of 1% Triton X-100 in 100% ethanol. Hepatic and serum triglyceride and free fatty acid concentration was determined by using triglyceride (Pointe Scientific, Inc, MI) and free fatty acid (Wako Chemicals USA, Inc, VA) reagent kits.

Hepatic microsomal preparation and *in vitro* acetaminophen glucuronidation. Liver tissue (~200 mg) was homogenized in 154 mM potassium chloride buffer containing 50 mM Tris-HCl and 1 mM EDTA (pH 7.4, containing protease inhibitor). Homogenates were centrifuged at 10,000 x g for 30 minutes at 4 °C. The supernatant was collected and centrifuged at 100,000 x g for 1 hour at 4 °C. Then the microsomal pellet was resuspended in 2 mL of 100 mM sodium pyrophosphate containing 0.1 mM EDTA (pH 7.4), centrifuged at 100,000 x g at 4 °C for

another 1 hour. The resulting supernatant was discarded and the microsomal pellet was resuspended with 100 μ L of 250 mM sucrose and stored at -80 $^{\circ}$ C.

Acetaminophen glucuronidation was determined using a modified method (Alkharfy and Frye, 2001). Briefly, 0.1 M sodium phosphate buffer (pH 7.8), 5 mM acetaminophen, 10 mM $MgCl_2$, 0.5% Brij58 (microsomal activator), and microsomes (10-50 μ g total protein) were preincubated in glass test tubes for 10 minutes. Then 4 mM UDP-GA was added to the solution to a total volume of 250 μ L. The tubes were placed in a 37 $^{\circ}$ C water bath and the reaction was started. The reaction was stopped after 60 minutes incubation by the addition of 25 μ L of 6% perchloric acid containing 25 μ g paraxanthine (internal standard), followed by vortexing and cooling on ice. Tubes were then centrifuged at 3,200 x g for 10 minutes at 4 $^{\circ}$ C, and the resulting supernatant was transferred into autosampler vials. Then 40 μ L aliquots of the supernatant were injected into the HPLC column; each measurement was repeated three times.

Measurement of DNA binding of transcriptional factors in mouse liver. The *in vitro* binding activity of transcriptional factors, namely aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), peroxisome proliferator-activated receptor (PPAR), pregnane X receptor (PXR), and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) to consensus cis-response elements was determined using a Procarta TF 9-plex custom arrays from Affymetrix (CA,USA) according to the manufacturer's instructions (Yaoi et al., 2006). Nuclear extracts were isolated from livers of C57BL/6J and ob/ob mice by using NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific, IL). The PPAR assays determine the binding of TFs to the consensus PPAR cis-response elements and thus do not differentiate the DNA binding of PPAR α from other PPAR family members, including PPAR β and PPAR γ . Samples were analyzed using a Luminex Bio-plexTM 200 Array reader with Luminex 100 X-MAP technology, and data were

acquired using Bio-Plex Data Manager Software Version 5.0 (Bio-Rad). All the data were normalized to TFIID binding activity.

Statistical analysis. Two-way analysis of variance (ANOVA) was used with genotype and fasting as the main factors, followed by Student–Newman–Keuls comparisons to assess the differences between groups ($P<0.05$). The data of *in vitro* DNA binding of transcriptional factors assays were analyzed by using unequal variance *t-test* with significance set at $P<0.05$.

Results:

Body and liver weight, serum glucose, triglyceride, free fatty acid, insulin, and leptin levels in C57BL/6J and ob/ob mice after fasting. Table 1 illustrated basic parameters of C57BL/6J and ob/ob mice after fasting. The fed ob/ob mice were 2 times heavier than C57BL/6J mice. After 24 hours fasting, ob/ob mice lost only 0.2% of BW; however C57BL/6J mice lost 17.3%. Ob/ob mice exhibited fatty liver, with liver weight being 1.2 fold higher than C57BL/6J mice. Fasting decreased liver weight by 28.7% in C57BL/6J mice (from 1.08 ± 0.02 g to 0.77 ± 0.01 g), but only 8.3% in ob/ob mice (from 2.40 ± 0.06 g to 2.20 ± 0.10 g). Serum glucose levels decreased after fasting in both lean and ob/ob mice. However in ob/ob mice, even after fasting, serum glucose levels were still elevated, being higher than that of C57BL/6J mice fed *ad libitum* (300.9 ± 36.7 mg/dL vs. 204.6 ± 2.5 mg/dL). Fasting increased serum free fatty acid (FFA) concentrations by 35.1% in C57BL/6J mice, and 22.4% in ob/ob mice. The insulin concentrations decreased from 1.37 ± 0.24 ng/mL to 0.19 ± 0.02 ng/mL in lean mice, and from 9.77 ± 0.50 ng/mL to 3.47 ± 0.45 ng/mL in obese mice, respectively. Serum leptin concentrations were measured in lean mice (556 ± 109 pg/mL). Fasting decreased serum leptin levels to undetectable concentrations.

Morphological changes in liver after fasting. Hematoxylin and eosin staining displayed accumulation of fat in ob/ob liver. C57BL/6J mice exhibited normal before and/or after fasting treatment by histopathological observation (Fig. 1A and 1B). In ob/ob mice, fat deposition was clearly associated with the perivenous area of liver lobules resulting in enlarged fat-laden hepatocytes in these parts of the liver (Fig. 1C and 1D). No hepatic necrosis, inflammation or fibrosis was present in either strain. To quantify hepatic fat accumulation in C57BL/6J and ob/ob mice, lipids were extracted from liver tissues and the content was quantified by using a

spectrophotometric method. Ob/ob mice exhibited higher hepatic triglyceride content, and fasting significantly induced hepatic triglyceride accumulation in both genotypes (Fig. 1E). Hepatic free fatty acid concentration increased significantly by fasting in C57BL/6J and ob/ob mice, however no significant difference were observed between these two genotypes in *ad libitum* or fasted status (Fig. 1F).

Fasting increased Ugt1a mRNA expression in mouse liver. The Ugt isoforms chosen to be measured was determined based on reported expression and inducibility in livers (Buckley and Klaassen, 2009). Fig. 2 illustrated fasting-induced expression of various Ugt1a isoforms, including Ugt1a1, 1a6, 1a7, and 1a9 in mouse liver. Fasting increased Ugt1a1 expression more than two fold in livers of C57BL/6J mice (Fig. 2A). Ob/ob mice fed *ad libitum* exhibited higher Ugt1a1 expression levels than C57BL/6J mice. After fasting, ob/ob mice showed even higher expression levels of Ugt1a1. Ugt1a6 regulation displayed the similar pattern (Fig. 2C), was induced 73% and 47% in C57BL/6J and ob/ob mice by fasting, respectively. And after fasting, ob/ob mice exhibited even higher expression levels. Ugt1a7 expression levels increased 56% and 45% by fasting in C57BL/6J and ob/ob mice, respectively. No significant difference of was observed between C57BL/6J and ob/ob mice in *ad libitum* or fasted status (Fig. 2D). Ugt1a9 expression levels were induced 233% in C57BL/6J mice by fasting. The expression levels increased 103% in ob/ob than C57BL/6J mice, and increased more by fasting. After fasting, ob/ob mice exhibited even higher expression levels of Ugt1a9 (Fig. 2E). No significant difference of Ugt1a2 expression was found in C57BL/6J and ob/ob mice by fasting (Fig. 2B). These data demonstrated obesity and fasting induced Ugt1a1, 1a6, 1a7, and 1a9 expression in mouse liver.

Fasting increased Ugt2b mRNA expression in mouse liver. Ugt2b1 expression increased 63% in C57BL/6J mice by fasting, more than 200% induction was found by fasting in ob/ob mice. Ob/ob mice showed lower Ugt2b1 expression levels after fasting (Fig. 3A). Ugt2b5 expression increased significantly in C57BL/6J and ob/ob mice livers after fasting (58% and 39%, respectively). And ob/ob mice exhibited significantly lower expression of Ugt2b5 gene after fasting (Fig. 3B). Fasting induced Ugt2b34, 2b35 and 2b36 expression slightly, with no significant difference in C57BL/6J mice, and induced 41% of Ugt2b34 expression in livers of ob/ob mice (Fig. 3C, 3D, and 3E).

Fasting increased Ugt2a3 and Ugt3a1/2 mRNA expression in mouse liver. Regarding basal expression, hepatic Ugt2a3 expression is significantly higher in ob/ob mice compared to C57BL/6J mice. Fasting increased Ugt2a3 expression by 100% in livers of C57BL/6J mice, but only 34% in livers of ob/ob mice (Fig. 4A). Fig. 4B illustrated Ugt3a1 expression increased 84% in ob/ob mice compared to C57BL/6J mice. Fasting induced Ugt3a1 expression 130% in C57BL/6J mice, and 79% in ob/ob mice. Compared to C57BL/6J mice, ob/ob mice exhibited higher expression levels of Ugt3a1 after fasting. Ugt3a2 expression levels in ob/ob mice were approximately two times higher than C57BL/6J mice. Fasting induced Ugt3a2 mRNA expression 160% in livers of C57BL/6J mice, but only 84% in ob/ob mice.

Fasting decreased hepatic acetaminophen glucuronidation activity *in vitro*. The current data demonstrate mRNA expression of various Ugt isoforms corresponded with obesity and fasting-induced steatosis. However, it is necessary to examine whether steatosis and fasting impact UGT activity. Fig. 5 illustrated that acetaminophen glucuronidation was 47% higher in ob/ob mice than C57BL/6J mice when fed *ad libitum*, implying that hepatic steatosis increased acetaminophen glucuronidation. Fasting decreased acetaminophen glucuronidation 30% in

C57BL/6J mice, but only 9% in ob/ob mice. The data demonstrated obesity or hepatic steatosis induced UGT activity, but fasting or food deprivation suppressed UGT activity, further demonstrated ob/ob mice were resistant to fasting effects.

Fasting increased AhR, CAR, PPAR α , PXR, Nrf2 and PGC1 α expression in mouse liver.

Fig. 6A illustrated that fasting increased AhR expression by 86% and 35% in C57BL/6J and ob/ob mice, respectively. Ob/ob mice exhibited higher expression levels of CAR in livers when fed *ad libitum*. After fasting, CAR expression levels increased more than 200% in C57BL/6J mice, and 91% in ob/ob mice (Fig. 6B). Fig. 6C illustrated ob/ob mice had higher expression levels of PPAR α compared to C57BL/6J mice; the mRNA expression levels were induced 139% and 140% in C57BL/6J and ob/ob mice, respectively. Compared to C57BL/6J mice, ob/ob mice exhibited higher expression levels of PPAR α after fasting. PXR expression was higher in livers of ob/ob mice fed *ad libitum* compared to C57BL/6J mice. After fasting, PXR expression levels increased by 82% in C57BL/6J mice, and 42% in ob/ob mice, and ob/ob mice showed higher expression levels (Fig. 6D). Nrf2 expressed higher in livers of ob/ob mice compared to C57BL/6J mice when fed *ad libitum*. Fasting doubled Nrf2 expression levels in livers of C57BL/6J mice, and 91% in ob/ob mice. Ob/ob mice exhibited the highest expression levels of Nrf2 after fasting (Fig. 6E). Fig. 6F illustrated PGC1 α expression levels increased dramatically in C57BL/6J mice after fasting (more than 6 fold), whereas this increasing was attenuated in ob/ob mice (only 190%).

Fasting activated transcriptional factor pathways in mouse livers. To determine whether fasting will activate each transcriptional factor signaling pathway, the specific target gene of each transcriptional factor was determined by quantitative real-time PCR. Cyp1a1 expression, the marker of AhR activation, was significantly induced by fasting in C57BL/6J mice (Fig. 7A).

And ob/ob mice exhibited higher expression levels of Cyp1a1. The CAR target gene, Cyp2b10, increased by 52 fold and 3.6 fold in C57BL/6J and ob/ob mice by fasting, respectively (Fig. 7B). Cyp4a10, the target gene of PPAR α , increased 10 fold and 0.5 fold by fasting in C57BL/6J and ob/ob mice, respectively (Fig. 7C). Fasting activated PXR pathway regarding the induction of the target gene expression of Oatp2 (Fig. 7D). Nqo1 and Gclc, the two Nrf2 target genes, showed similar expression patterns to that of Nrf2, excluded no significant induction of Gclc by obesity. Correspondingly, fasting increased Nqo1 expression 300% in C57BL/6J mice, only 63% in ob/ob mice (Fig. 7E). Gclc increased 46% in C57BL/6J mice, and 68% in ob/ob mice (Fig. 7F). These data demonstrated the transcriptional factor pathway of AhR, CAR, PPAR α , PXR, and Nrf2 were likely activated in hepatic steatosis caused by obesity and/or fasting.

Cyclic AMP (cAMP) induced Ugt expression in mouse primary hepatocytes isolated from C57BL/6 and ob/ob mice. Fasting promotes glucagon secretion into the blood, subsequently increases hepatic intercellular cAMP concentrations, and upregulates gluconeogenesis. A previous report demonstrated cAMP treatment increased Ugt1a1 in mouse hepatocytes (Ding et al., 2006). Thus, other Ugt isoforms, which were increased *in vivo* were also examined. Primary hepatocytes isolated from mouse liver were treated with 8-Br-cAMP, the analogue of natural signal molecule cyclic AMP for 24 hours. Expression of Ugt1a1, 1a2, 1a6, 1a7, 1a9, 2b1, 2b5, 2b34-36, 2a3, 3a1, and 3a2 significantly increased after the treatment in hepatocytes from C57BL/6J and ob/ob mice, demonstrated the signaling pathway activated by cAMP played vital roles in Ugt induction in mouse primary hepatocytes (Fig. 8).

Fasting increased DNA binding of transcriptional factors to their cis-response elements.

TF Procarta transcription factor binding assays were used to measure nuclear transcription factor levels via *in vitro* binding activity to consensus cis-response elements. In lean mice, DNA

binding of CAR, PPAR, and Nrf2 were induced 77%, 79%, and 75% by fasting, respectively. A trend for increased AhR and PXR binding was observed but not statistically significant. However in obese mice, DNA binding of these transcriptional factors remained unchanged after fasting, except AhR DNA binding activity was attenuated, further suggested ob/ob mice were resistant to fasting effects (Fig. 9).

Discussion:

UDP-glucuronosyltransferases are highly expressed in liver and intestine, and responsible for glucuronidation of chemicals ranging from hormones, bile acids, drugs, and of ones of environmental exposure. Glucuronidation influences the pharmacokinetic profiles of the drugs in tissues with high glucuronidation capacity. The study herein illustrates two key findings – the expression of multiple Ugt isoforms is associated with fatty liver and after fasting. Both conditions correspond with increased hepatic triglyceride content, increased nuclear receptor expression and binding (Supplemental Table S2).

Fasting is known to increase glucagon secretion and hepatic intracellular adenosine 3',5'-monophosphate (cAMP) levels, has been shown to induce Ugt1a1 expression through a network of cAMP, PGC1 α , HNF4 α and CAR signaling pathways (Ding et al., 2006). In response to fasting induction, serum epinephrine and glucagon concentrations increase, which will increase intracellular cAMP levels, then activates PKA signaling pathway, which upregulates downstream genes expression of PGC1 α and PPAR α , and subsequently induces Ugt target genes expression (Fig. 10). Our data demonstrate additional Ugt1 and Ugt2 isoforms increased after food deprivation and in primary mouse hepatocytes treated with cAMP analogue (8-Br-cAMP), suggesting enhanced intracellular cAMP levels play vital roles in the induction of Ugt isoforms by fasting.

Mice have relatively high expression levels of Ugt1a1 and 1a6 in liver (Shelby et al., 2003). In the current study, obesity- and fasting-induced steatosis increased Ugt1a1 and 1a6 mRNA expression along with an observed increasing of CAR binding and a trend toward increasing of PXR binding. Multiple studies have illustrated prototypical CAR and PXR activators increased Ugt1a1 and 1a6 mRNA expression in mouse liver via CAR- and PXR-

dependent mechanism (Buckley and Klaassen, 2009; Ou et al., 2010). Additionally, rifampicin, a human PXR activator, increased UGT1A1 mRNA and protein expression in HepG2 cells, and dexamethasone enhanced PXR-mediated induction (Sugatani et al., 2005). Transgenic mice of overexpressing a constitutively active form of human PXR exhibited significant induction of Ugt1a1 and 1a6 expression in the liver, suggested the induction of Ugt expression via PXR-dependent pathway (Ou et al., 2010). Our results demonstrate mRNA levels of CAR and PXR upregulated in livers of ob/ob mice or after fasting (Fig. 6B and 6D), and the activation of signaling pathway of CAR and PXR (Fig. 7B and 7D), which would promote Ugt expression in mouse liver subsequently.

PPAR α , a key regulator of fatty acid oxidation and lipid homeostasis, is essential for adaptation to fasting in rats and mice (Leone et al., 1999). Like CAR and PXR activators, PPAR α activators induced Ugt1 isoforms expression in mouse and rat liver, as well as in human hepatocytes. PPAR-responsive elements were identified in the promoter of UGT1A1, 1A3, and 1A6 genes, which mediated PPAR-dependent UGT induction in HepG2 cells (Senekeo-Effenberger et al., 2007). Similarly, Ugt2 isoforms were inducible by PPAR α ligands in mouse liver and human hepatocytes (Barbier et al., 2003). It is well documented that PPAR α activity is upregulated in steatosis and livers from ob/ob mice (Memon et al., 2000). The current research also demonstrates PPAR α mRNA expression increased in steatotic livers of ob/ob mice and fasting-induced steatosis, suggesting PPAR α activation play an important role in the observed Ugt isoforms induction.

Nrf2 is considered to be a major regulator of cytoprotective detoxification and antioxidant enzymes expression (Wakabayashi et al., 2010). Enhanced Nrf2 activity and expression following electrophilic and oxidant exposure has been termed as the antioxidant

response, inducing phase I and II detoxification enzymes, namely UDP-glucuronosyltransferases. It is well described that Nrf2 activators can upregulate certain UGT1 and UGT2 isoforms in human cell lines (Yueh and Tukey, 2007), as well as in mouse and rat liver and intestine (Buckley and Klaassen, 2009). Ugt1a6 and 1a7 induction by oltipraz was abrogated in Nrf2-null mice, suggesting a major role for Nrf2 in the induction of Ugt expression (Iida et al., 2004). Ugt1a6 expression was inducible in mouse liver after constitutive Nrf2 activation via silencing Kelch-like ECH-associated protein 1 (Keap1-knockdown) (Reisman et al., 2009), consistent with the latter findings of Nrf2 activation upregulated Ugt1a6 expression pharmacologically (Buckley and Klaassen, 2009). Data from our current study illustrate increased hepatic Nqo1 and Gclc expression levels in ob/ob and fasted mice, suggesting Nrf2 signaling activated by different models of steatosis, which could be responsible for the Ugt induction in mouse liver.

The existence of Ugt3 subfamily isoforms was first noted in 2000 (Tukey and Strassburg, 2000) after homology searching of the databases assembled as part of the Human Genome Project (Radominska-Pandya et al., 1999). In contrast to the extensive studies on the function of Ugt1 and Ugt2 subfamilies, limited studies that describe the catalytic properties and substrates of the Ugt3 subfamily and regulation of gene expression remain largely undescribed. Ugt3a1/2 is expressed at relatively high levels in kidney, low levels in liver, and was not detectable in lung, stomach, brain, gonad, and placental tissue (Buckley and Klaassen, 2007). Ugt3a2 uses both UDP glucose and UDP xylose as a glucose donor, and is relatively low in liver and gastrointestinal tract, but higher in thymus, testis and kidney, suggesting that it likely plays a minor role in drug metabolism (MacKenzie et al., 2010). In the current study, Ugt3a1 and 3a2 mRNA levels increased in livers from ob/ob and fasted mice. Further research on Ugt3

subfamily is needed to reveal significant therapeutic and/or toxicological implications in the future.

Our current data demonstrate fasting increased the mRNA levels of additional Ugt isoforms, but decreased UGT activity *in vitro* in mouse liver. UGT activity *in vivo* is dependent on hepatic glycogen stores (Banhegyi et al., 1988). During severe food deprivation, the UDP-glucose supply might be insufficient for acetaminophen conjugation despite the induction of Ugt isoforms in liver. However, it is also known that fasting decreases the rate of protein synthesis and increases protein degradation in mammalian animal models (Cherel et al., 1991), which subsequently decrease cellular protein levels described by decreased UGT activity from the current study, even despite higher mRNA expression. Historically, it has been established that fasting increased acetaminophen hepatotoxicity in rodents (Walker et al., 1982). It is well known that induction of acetaminophen toxicity in rodents have a circadian component and timing of acetaminophen dosing is critical for the level of hepatotoxicity that manifests (Schnell et al., 1984). It should be noted that cases of acetaminophen-induced hepatotoxicity in humans (especially children) has been documented with concomitant fasting and malnutrition (Fernando and Ariyananda, 2009). In the present study, we observed discordant Ugt expression and acetaminophen glucuronidation. Fasting increased Ugt mRNA isoforms likely to carry out acetaminophen biotransformation, such as Ugt1a1, 1a5, and 1a6 (Kessler et al., 2002). However, the present data illustrate that fasting decreased acetaminophen glucuronidation activity in lean mice, but minimally did so in *ob/ob* mice indicating that extra fat stores may provide resistance. Additionally, in *ob/ob* mice, obesity-induced hepatic steatosis increased Ugt expression and acetaminophen glucuronidation *in vitro* activity. Obesity has been associated with enhanced capacity for biotransformation of lorazepam, oxazepam, and acetaminophen, which were via

glucuronide conjugation (Abernethy et al., 1983). Together, the findings indicate that the obese population could be resistant to acetaminophen induced liver injury under normal conditions, as well as when food consumption has been limited due to illness.

It is interesting that despite such different models, Ugt isoforms and nuclear receptor expression levels corresponded with hepatic triglyceride content. In the current experiment, certain Ugt isoforms (Ugt1a1, 1a6, 1a9, 2a3, 2b34, 3a1, and 3a2) corresponded with hepatic triglyceride content (Supplemental Figure. S1), suggesting excessive triglyceride in hepatic steatosis plays a vital role in upregulating nuclear receptors and induces Ugt expression. The increased hepatic triglyceride content was also associated with increased nuclear receptor expression and binding activity. Briefly, our study and others have observed that fasting and obesity caused increased hepatic triglyceride content (Newberry et al., 2003). Both conditions are associated with increased levels of circulating free fatty acid and increased hepatic steatosis (Guan et al., 2009). This increase in hepatic steatosis is accompanied by the increase in expression and binding activity of nuclear receptors, such as AhR, CAR, Nrf2, PXR, and PPAR α , which are known to modulate Ugt expression directly or indirectly through interactions with other nuclear receptors (such as HNFs) (Osabe et al., 2008). However, because food deprivation is known to induce protein degradation and repress protein synthesis, hepatic UGT isoform expression was likely decreased at the protein level, which corresponded to the decreased UGT activity (summarized in Figure 10).

Overall, the current study demonstrates obesity- and fasting-induced UDP-glucuronosyltransferases expression in mouse liver, and the induction was associated with AhR, CAR, PPAR α , PXR, Nrf2, and PGC1 α upregulation. Obesity increased hepatic glucuronidation activity; however fasting decreased the activity in C57BL/6J or ob/ob mice. Additionally, ob/ob

mice appeared to be resistant to fasting effects. These data indicate nuclear receptor and Ugt expression may be responsive to hepatic triglyceride concentrations, as well as blood glucose levels. Overall, there is potential for differential glucuronidation pharmacokinetics for drugs and environmental chemicals in the obese and fasted liver, which warrants further investigation.

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Footnotes:

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Legends for figures:

Figure 1. Representative hematoxylin and eosin (HE) staining and hepatic lipid accumulation of C57BL/6J and ob/ob mice after fasting. A-D, Liver samples from adult male C57BL/6J (C57) and ob/ob (ob/ob) mice fed *ad libitum* (Fed) or withheld food (Fasted) for 24 hours were assessed by hematoxylin and eosin (HE) staining. Magnification: 200 \times . Scale bar: 50 μ m (n=4 per group). E-F, Hepatic lipids were extracted from liver tissues and hepatic triglyceride and free fatty acid were measured for quantitative lipid content. Data represent average \pm S.E. (n=6~8 per group). Groups without a common letter are significantly different ($P<0.05$).

Figure 2. Fasting induced Ugt1a1, 1a2, 1a6, 1a7, and 1a9 in mouse liver. Total RNA was isolated from livers of lean (C57) or obese (ob/ob) mice that were fed *ad libitum* (Fed) or withheld food (Fasted) for 24 hours, and mRNA levels were quantified by quantitative real-time PCR. Data represent average \pm S.E. (n=5~8 per group). All data were normalized to 18S rRNA levels. Groups without a common letter are significantly different ($P<0.05$).

Figure 3. Fasting induced Ugt2b1, 2b5, 2b34, 2b35, and 2b36 in mouse liver. Total RNA was isolated from livers of lean (C57) or obese (ob/ob) mice that were fed *ad libitum* (Fed) or withheld food (Fasted) for 24 hours, and mRNA levels were quantified by quantitative real-time PCR. Data represent average \pm S.E. (n=5~8 per group). All data were normalized to 18S rRNA levels. Groups without a common letter are significantly different ($P<0.05$).

Figure 4. Fasting induced Ugt2a3, 3a1, and 3a2 in mouse liver. Total RNA was isolated from livers of lean (C57) or obese (ob/ob) mice that were fed *ad libitum* (Fed) or withheld food (Fasted) for 24 hours, and mRNA levels were quantified by quantitative real-time PCR. Data represent average \pm S.E. (n=6~8 per group). All data were normalized to 18S rRNA levels. Groups without a common letter are significantly different ($P<0.05$).

Figure 5. Effect of obesity and fasting on acetaminophen glucuronidation *in vitro* in C57BL/6J and ob/ob mice. Hepatic microsomes were isolated from livers of C57BL/6J (C57) or obese (ob/ob) mice that were fed *ad libitum* (Fed) or withheld food (Fasted) for 24 hours, and acetaminophen glucuronidation *in vitro* was measured by using HPLC. Data represent the average \pm S.E. (n=8 per group). Groups without a common letter are significantly different ($P<0.05$).

Figure 6. Obesity and fasting increased AhR, CAR, PPAR α , PXR, Nrf2, and PGC1 α mRNA expression in mouse liver. Total RNA was isolated from livers of C57BL/6J (C57) or obese (ob/ob) mice that were fed *ad libitum* (Fed) or withheld food (Fasted) for 24 hours, and mRNA levels were quantified by quantitative real-time PCR. Data represent average \pm S.E. (n=6~8 per group). All data are normalized to 18S rRNA levels. Groups without a common letter are significantly different ($P<0.05$).

Figure 7. Obesity and fasting induced Cyp1a1, Cyp2b10, Cyp4a10, Oatp2, Gclc, and Nqo1 mRNA expression in mouse liver. Total RNA was isolated from livers of C57BL/6J (C57) or obese (ob/ob) mice that were fed *ad libitum* (Fed) or withheld food (Fasted) for 24 hours, and

mRNA levels were quantified by quantitative real-time PCR. Data represent average \pm S.E. (n=6~8 per group). All data were normalized to 18S rRNA levels. Groups without a common letter are significantly different ($P<0.05$).

Figure 8. 8-Br-cAMP induced Ugt isoforms mRNA expression levels in hepatocytes.

Primary mouse hepatocytes were isolated from 2-3 month old C57BL/6J and ob/ob mouse livers and seeded on collagen-coated 6-well plates. After seeding for 24 hours, cells were treated with vehicle (CON) or 0.1 mM 8-Br-cAMP (cAMP) for 24 hours. Total RNA was isolated and mRNA relative levels were quantified by quantitative real-time PCR. Data represent average \pm S.E (n=3). All data were normalized to 18S rRNA levels. Groups without a common letter are significantly different ($P<0.05$)

Figure 9. Steatosis and fasting modulate nuclear AhR, CAR, PPAR, PXR and Nrf2 binding.

Nuclear extraction from livers of C57BL/6J (C57) and ob/ob (ob/ob) mice (n=3 per group) were used to determine *in vitro* DNA binding of transcriptional factors with Procarta TF 9-plex custom arrays. Data represent average \pm S.E. All data were normalized to TFIID values. *, #, \$, $P<0.05$, C57-Fasted compared C57-Fed, ob/ob-Fasted compared C57-Fasted, and ob/ob-Fasted compared ob/ob-Fed, respectively.

Figure 10. Summary of obesity and fasting-induced Ugt induction. Obesity and food deprivation induced steatosis, which was accompanied by increased hepatic triglyceride and free fatty acid content, and subsequently induced the network of transcriptional factors of AhR, CAR, Nrf2, PXR, and PPAR α , and then increased Ugt isoform mRNA expression. Fasting upregulates

PKA signaling along with PGC1 α and HNF4 α , also induced the network of transcriptional factors of AhR, CAR, Nrf2, PXR, and PPAR α , and then induced Ugt isoform mRNA expression. Food deprivation decreased protein synthesis and increased protein degradation, which decreased UGT activity in livers.

Table 1. Characters and metabolic parameters in 8-wk-old C57BL/6J and ob/ob mice after fasting.

	C57		ob/ob	
	Fed	Fasted	Fed	Fasted
Body Weight (BW), g	21.64±0.15 ^a	17.90±0.34 ^b	38.18±0.40 ^c	38.09±1.18 ^c
Liver Weight (LW), g	1.08±0.02 ^a	0.77±0.01 ^b	2.40±0.06 ^c	2.20±0.10 ^d
LW/BW, %	4.99±0.07 ^a	4.28±0.05 ^b	6.27±0.10 ^c	5.76±0.08 ^d
Glucose, mg/dL	204.6±2.5 ^a	178.7±7.2 ^a	699.9±24.2 ^b	300.9±36.7 ^c
TG, mg/dL	43.01±6.53 ^a	28.56±1.08 ^b	49.94±5.50 ^a	45.00±2.18 ^a
FFA, mEq/dL	0.34±0.01 ^a	0.50±0.03 ^b	0.67±0.05 ^c	0.82±0.06 ^d
Insulin, ng/mL	1.37±0.24 ^a	0.19±0.02 ^b	9.77±0.50 ^c	3.47±0.45 ^d
Leptin, pg/mL	556±109	n.d.	n.d.	n.d.

Adult male C57BL/6J and ob/ob mice were fed *ad libitum* (Fed) or withheld food (Fasted) for 24 hours. Serum was collected. Serum glucose, triglyceride and free fatty acid were measured by a spectrophotometric assay; hormones were measured by a luminex-based assay. Data represent average ± S.E. (n=4 to 8 per group). Groups without a common letter are significantly different ($P<0.05$). n.d., not detected.

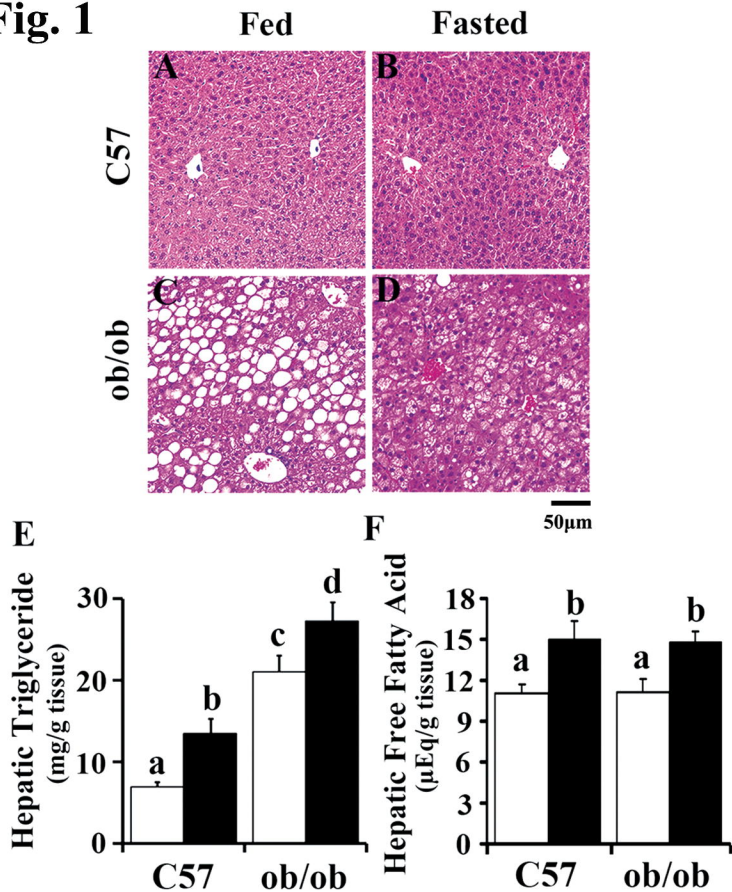
Fig. 1

Fig. 2

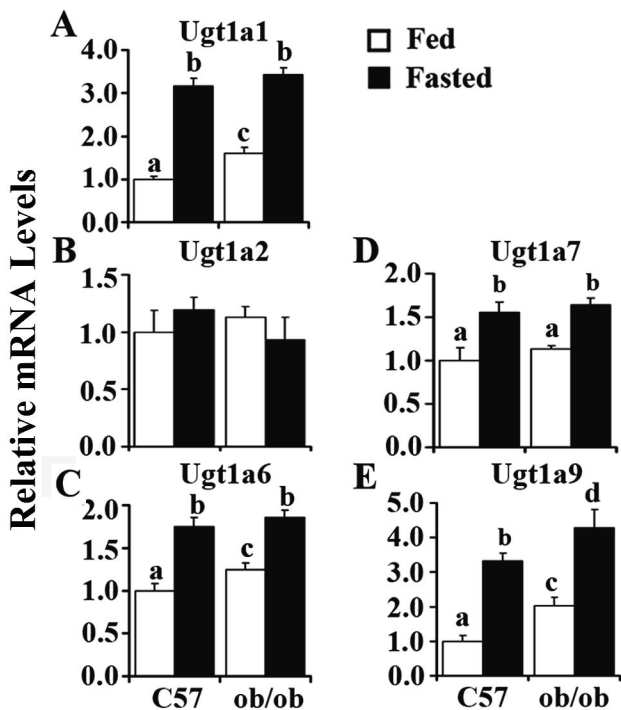


Fig. 3

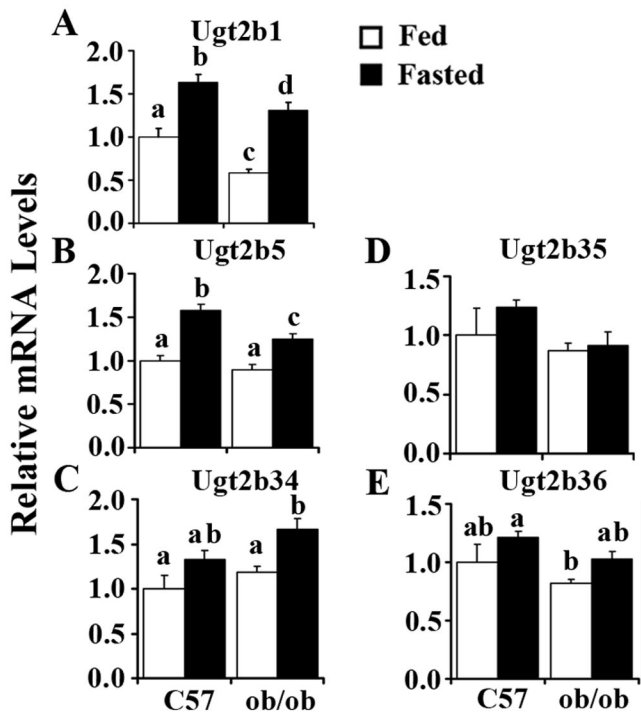


Fig. 4

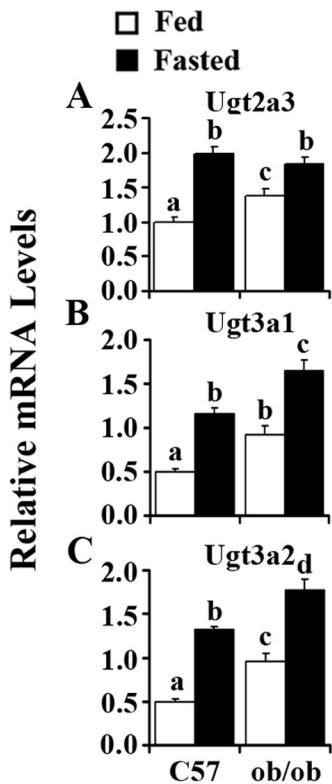


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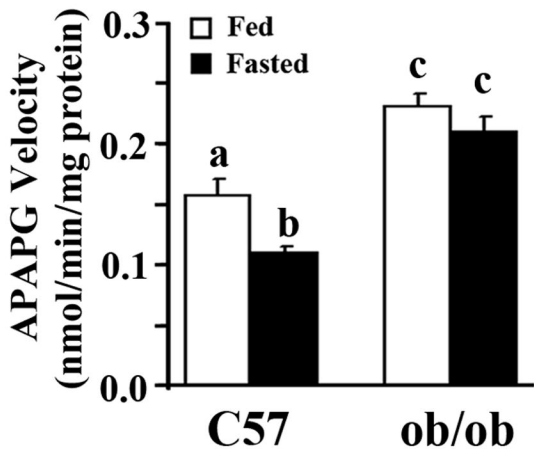


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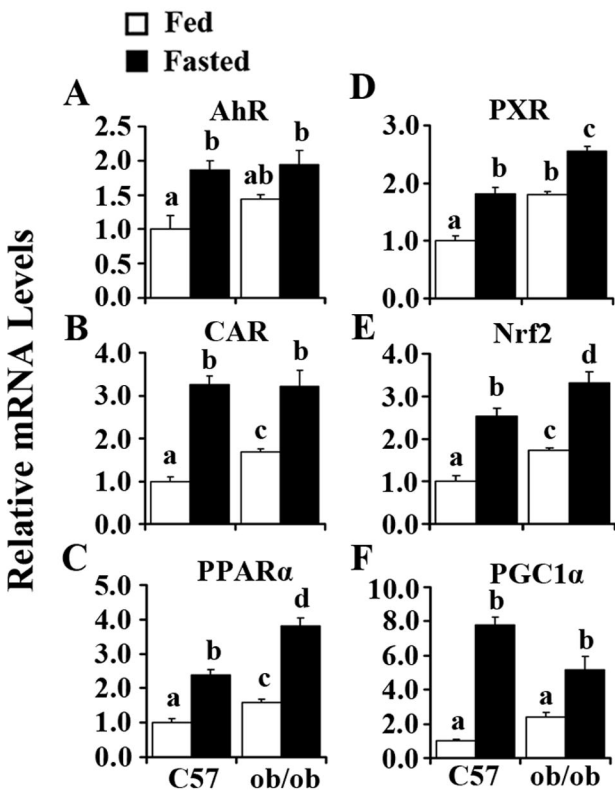


Fig. 7

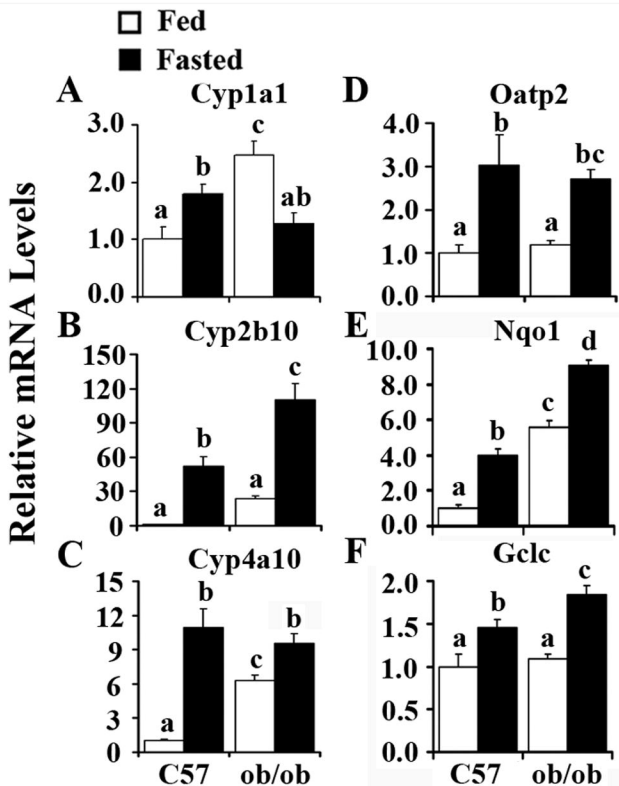


Fig. 8

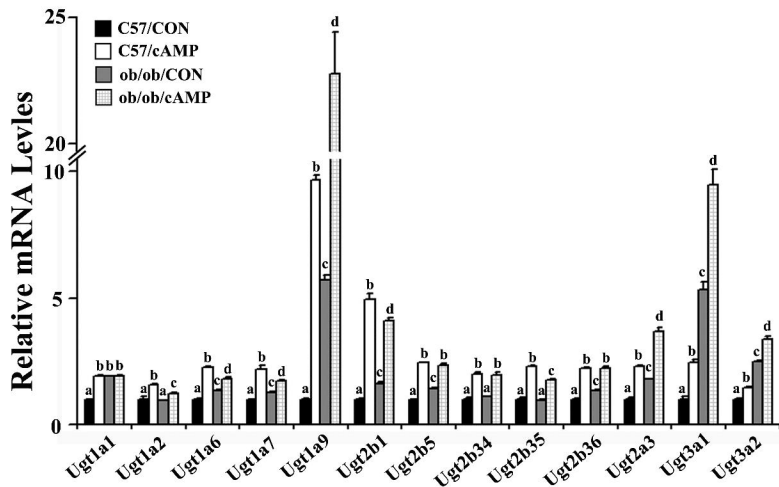


Fig. 9

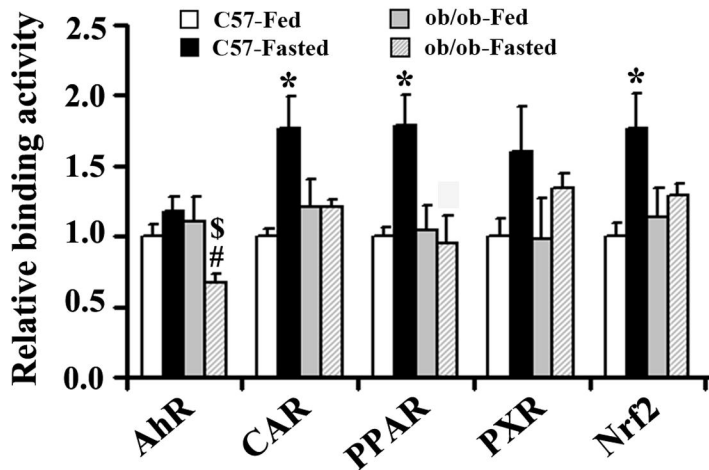


Fig. 10

