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Drug-Metabolizing Enzyme and Transporter Expression in a Mouse Model of Diabetes and Obesity

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Abstract: Obesity and type II diabetes pose a serious human health risk. Obese or diabetic patients usually take prescription drugs that require hepatic and renal metabolism and transport, and these patients sometimes display different pharmacokinetics of these drugs. Therefore, mRNA and protein expression of drug-metabolizing enzymes (DMEs) and transporters was measured in livers and kidneys of adult wild-type and ob/ob mice, which model obesity and diabetes. mRNA expression of numerous DMEs increased by at least 2-fold in livers of male ob/ob mice, including Cyp4a14, Cyp2b10, NAD(P)H:quinone oxidoreductase 1 (Nqo1), and sulfotransferase 2a1/2. In general, expression of uptake transporters was decreased in livers of ob/ob mice, namely organic anion-transporting polypeptides (Oatps) and sodium/taurocholate cotransporting polypeptide (Ntcp). In particular, Oatp1a1 mRNA and protein expression in livers of ob/ob mice was diminished to <5% and <15% of that in wild-types, respectively. Generally, the mRNA and protein expression of efflux transporters multidrug resistance-associated proteins (Mrps) was increased in livers of ob/ob mice, particularly with Mrp4 expression being elevated by at least 6-fold and Mrp2 expression at least 3-fold in livers of ob/ob mice. In kidney, Nqo1, Mrp3, 4, Oatp1a1, and organic anion transporter 2 (Oat2) showed significant alterations with mRNA expression levels in ob/ob mice, being increased for Nqo1 and Mrp4 and decreased for Mrp3, Oatp1a1, and Oat2. In summary, the expression of a number of DMEs and transporters was significantly altered in livers and kidneys of ob/ob mice. Since expression of some DMEs and transporters is regulated similarly between mouse and human, the data from this study suggest that transporter expression in liver and kidney may be changed in patients presenting with obesity and/or type II diabetes.

Keywords: Drug-metabolizing enzymes; transporters; ob/ob mice; obesity; diabetes; organic anion-transporting polypeptide; Oatp1a1; multidrug resistance-associated protein; Mrp4

Introduction

Obesity, which has become one of the greatest threats to global human health, shows significant association with type II diabetes and other metabolic disease clusters, such as fatty liver disease, high blood pressure, and high cholesterol levels.[1–3] More than 80% of people with type II diabetes are overweight.[4] The linkage between obesity and diabetes has become clear in the past decade: low-level chronic and local inflammation, triggered by obesity, is critical in the

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development of type II diabetes.[5,6] Diabetic patients usually take prescription drugs that require hepatic and renal metabolism and transport, which are accomplished by drug-metabolizing enzymes (DMEs) and transporters in the body. Altered expression of DMEs and transporters results in changed drug pharmacokinetics and pharmacodynamics in humans and rodents. Pharmacokinetic changes of several drugs, such as acetaminophen, oltipraz, chlorzoxazone, theophylline, torasemide, clarithromycin, furosemide, and methotrexate, have been reported in rat models of diabetes mellitus induced by alloxan or streptozotocin treatment. [7–14] Changes in expression of cytochrome P450 (Cyp) isoforms were also observed in those rat models of diabetes, such as increased Cyp1A2, 2B1/2, 2E1, and 3A1(23) and decreased 2C11.[9–11] In rats, streptozotocin treatment also increased multidrug resistance protein 2 (Mdr2) in liver, resulting in increased phospholipid secretion into bile.[15] Although the causes and symptoms for diabetes associated with obesity differ from insulin-dependent type I diabetes, it is possible for altered expression of DMEs and transporters to occur in obesity and its associated diabetes and fatty liver, resulting in changed drug pharmacokinetics and pharmacodynamics. However, there is minimal data available regarding the expression of DMEs, hepatobiliary, and renal transporters in obesity and its associated diabetes and fatty liver.

DMEs are important for the detoxification of exogenous compounds including prescription drugs. Among CYP isoforms, CYP3A4 in humans is responsible for metabolism of approximately 60% of all clinically used drugs, as well as some steroids and bile acids. Compared to CYP3A4, CYP2B6 has a relatively smaller but still significant role in drug metabolism. Human CYP3A4 and 2B6 correspond to Cyp3a11 and 2b10 in mouse, respectively. Cyp4a14 and 2e1 in mouse are two key enzymes related to obese and diabetes through metabolizing fatty acids and endogenous ketones.[16] Besides Cyps, there are some other enzymes playing important roles for endogenous and exogenous compounds inactivation or detoxification. For instance, NAD(P)H: quinone oxidoreductase 1 (Nqo1) acts as a cytoprotectant by catalyzing obligate two electron reduction of quinones and semiquinones compounds to relatively stable hydroquinones, and sulfotransferases (Sults) lead to the formation of hydrophilic products for excretion through sulfoconjugation.[18] Heme oxygenase-1 (HO-1) exerts antioxidant cytoprotective effects by augmenting the breakdown of prooxidant heme to billirubin and facilitating iron extrusion from cells.[19]

Hepatobiliary transporters equip hepatocytes to extract compounds from portal blood and subsequently excrete them and their metabolites into bile or blood. Uptake solute carriers, including organic anion-transporting polypeptides (Oatps), sodium/taurocholate-cotransporting polypeptide (Ntcp), and organic cation transporters (Octs), are localized to the basolateral membrane and transport xenobiotics and bile acids into hepatocytes. Efflux transporters that are localized

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to the canalicular membrane mediate excretion of chemicals and their metabolites from hepatocytes into bile, whereas efflux transporters localized to the basolateral membranes mediate efflux of chemicals from hepatocytes into blood. Efflux transporters include multidrug resistance proteins (Mdrs), multidrug resistance-associated protein (Mrps), bile salt export pump (Bsep), and breast cancer resistance protein (Bcrp). In kidney, organic anions transporters (Oats), organic cation transporters (Octs), Oatps, Mdrs, and Mrps are important for determining the clearance and urinary excretion of many drugs and toxicants.

Proper modeling of obesity and diabetes in rodents will provide invaluable information to aid in the prediction of drug efficacy, pharmacokinetics, and toxicity in morbidly obese humans. In the current study, we intend to use the ob/ob mouse strain as a model to characterize expression of DMEs and transporters in obesity and its associated diabetes. Ob/ob mice were first described in 1994, as mice with a mutation in the obese gene that encodes for a protein called leptin.[20] The ob/ob phenotype displays profound obesity and type II diabetes, mimicking morbid obesity in humans.[20] Increased circulating cholesterol levels (2–3 fold over control after fasting), triglyceride levels (1.5- to 2-fold), and insulin levels (41.5 vs 0.8 ng/mL in 12-week old males) are observed in the ob/ob mice, with glucose tolerance and insulin resistance.[21,22] In addition, ob/ob mice display hepatic steatosis (fatty liver), which is a condition common to obesity and type II diabetes. Hepatic steatosis also shows association with changes of transporter expression in liver in some mouse models.[23] Given the prevalence of these diseases in the United States and the increasing use of prescription drugs, it is important to determine whether they can cause altered drug pharmacokinetics or drug−drug interactions. Therefore, we hypothesized that ob/ob mice exhibit altered mRNA and protein expression of hepatic and renal DMEs and transporters with subsequent changes in pharmacokinetics of some drugs. In the current study, we characterized mRNA and protein expression of numerous DMEs and transporters in livers and kidneys of ob/ob.

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Experimental Methods

Animals. Eight-week-old C57BL/6 and ob/ob (B6.V-Lep^{ob}/J) mice ($n = 5$ for each strain and gender) were purchased from the Jackson Laboratories (Bar Harbor, ME), housed under a constant dark/light cycle (12 h/12 h), and given food and water *ad libitum*. The mice were fed the same feed (LabDiet 5K20) as at Jackson Laboratories in order to maintain a consistent feed source. After 3 weeks acclimation, mice were anesthetized by isofluorane inhalation. Blood was collected by cardiac puncture, and serum was obtained after centrifugation. Livers and kidneys were collected, snap frozen with liquid nitrogen, and stored in −80 °C for future analysis. All animal experiments were approved by Institutional Animal Care and Use Committee (IACUC) of University of Rhode Island.

Glucose Assay. Glucose levels in serum were analyzed using glucose (HK) assay kit (Sigma-Aldrich, Saint Louis, MO) according to the manufacturer's instruction with a slight modification. Samples were analyzed using a 96-well microplate format, with10 μL of serum (0.5–50 μg of glucose) and 100 μL of glucose assay reagent added into each well. The light path length at which the absorbance was read in microplate was determined by taking the average ratio of M340/M340$_{corr}$ values, in which M340 = $A_{\text{reagent blank}} - A_{\text{DI blank}}$ in microplate and M340$_{corr}$ = $A_{\text{reagent blank}} - A_{\text{DI blank}}$ in 1 cm cuvettes.

Hematoxylin/Eosin and Oil Red O Staining. Standard methods were used to fix and stain liver tissues with hematoxylin and eosin. Briefly, liver tissues were fixed for 24 h in 10% formaldehyde and transferred to 75% ethanol, embedded with paraffin, sectioned to 5 μm, and then stained with hematoxylin and eosin. For Oil Red O staining, frozen liver tissues were sectioned to 5 μm thickness and then stained using Oil Red O kit (Diagnostic BioSystems, Inc. (DBS), Pleasanton, CA) as per the manufacturer's procedure.

RNA Extraction. Total RNA from liver or kidney was extracted using the RNA Bee reagent (Tel-Test Inc., Friendswood, TX) according to the manufacturer's protocol. RNA integrity was confirmed by formaldehyde−agarose gel electrophoresis.

Oligonucleotide Probe Sets for bDNA Assay. Probe sets for mouse Cyp4a14, 2b10, 3a11, 2e1, Ho-1, Nqo1, Sult2a1/2, Mrp1–6, Oatp1a1, 1a4, 1b2, 1a6, 2b1, Oat1–3, Bsep, Bcrp, Ntcp, and Mdr2 have been previously described.[24–30] Mouse gene sequences of interest were acquired from GenBank. Multiple oligonucleotide probe sets [capture extender (CE),

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label extender (LE), and blocker (BL) probes] were designed using ProbeDesigner software version 1.0 (Bayer Corp., Emeryville, CA), to be highly specific to a single mRNA transcript. All oligonucleotide probes were designed with a melting temperature of approximately 63 °C. Each probe designed in ProbeDesigner was submitted to the National Center for Biotechnological Information for nucleotide comparison by the basic local alignment search tool (BLASTn) to ensure minimal cross-reactivity with other mouse sequences. Oligonucleotides with a high degree of similarity to other mouse gene transcripts were eliminated from the design.

Branched DNA Signal Amplification (bDNA) Assay. All reagents for analysis (i.e., lysis buffer, capture hybridization buffer, amplifier/label probe buffer, and substrate solution) were supplied in the QuantiGene HV signal amplification kit (Panomics, Fremont, CA). Oligonucleotide probes were diluted in lysis buffer. Total RNA (1 μg/μL, 10 μL) was added to each well of a 96-well plate containing 50 μL of capture hybridization buffer and 100 μL of diluted probe set. Total RNA was allowed to hybridize to probe sets overnight at 53 °C. Subsequent hybridization steps were carried out according to the manufacturer's protocol, and luminescence was measured with a Quantiplex 320 bDNA luminometer interfaced with Quantiplex Data Management Software version 5.02 (Bayer Corp., Diagnostics Div., Tarrytown, NY). The luminescence for each well was reported as relative light units (RLU) per 10 μg total RNA.

Membrane, Cytosol, and Nuclear Fraction Preparation. Livers (~50 mg) or half of a kidney were homogenized in sucrose−Tris (ST) buffer (10× volume of sample amount, 0.25 mol/L sucrose, 10 mmol/L Tris–HCl, pH 7.4) containing protease inhibitor cocktail (2 μL/mL, Sigma Chemical Co. P8340) and centrifuged at 100000*g* for 60 min at 4 °C. The resulting supernatant was the cytosolic fraction and the pellet was the membrane fraction. ST buffer was used to resuspend the resulting pellet (membrane fraction). Nuclear proteins were prepared from liver using the NE-PER kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Membrane, cytosolic, and nuclear protein concentrations were determined by the method of Lowry using Bio-Rad protein assay reagents (Bio-Rad Laboratories, Hercules, CA).

Table 1. Information for Primary Antibodies Used for Western Blots

antigen	antibody	source	cell fraction	primary Ab concn	MW (kDa)
Cyp2e1	Ab28146	Abcam[a]	membrane	1:5000	~60
Cyp3a11	PA1–343	Affinity Bio[a]	membrane	1:5000	~45
Ho-1	SPA895	Stressgen[a]	membrane	1:2000	~32
Nqo1	Ab2346	Abcam[a]	cytosol	1:1000	~32
Ntcp	NtcpK4	Switzerland[b]	membrane	1:5000	~50
Oatp1		KUMC[c]	membrane	1:1000	~70
Oatp2		KUMC[c]	membrane	1:1000	~70
Oatp4		KUMC[c]	membrane	1:2000	~70
Mrp1	MRPr1	VUMC[d]	membrane	1:2000	~190
Mrp2	M_2III-5	VUMC[d]	membrane	1:600	~190
Mrp3	M_3II-2	VUMC[d]	membrane	1:2000	~180
Mrp4	M_4I-10	VUMC[d]	membrane	1:2000	~160
Mrp5	M_5I-60	VUMC[d]	membrane	1:50	~160
Mrp6	M_6II-68	VUMC[d]	membrane	1:1000	~165
Bcrp	BXP-53	VUMC[d]	membrane	1:2000	~75
HNF1α	H140	Santa Cruz[a]	nuclear	1:500	~79

[a] Abcam (Cambridge, MA), Affinity Bioreagents (Golden, CO), Stressgen (San Diego, CA), Santa Cruz Biotechnologies (Santa Cruz, CA). [b] University of Hospital, Zurich, Switzerland, Bruno Steiger. [c] University of Kansas Medical Center, Kansas City, KS, Curtis D. Klaassen. [d] VU Medical Center, Amsterdam, The Netherlands, George Scheffer.

Western Analysis of Mouse Liver Fractions. Methods for Western blot analysis of transport and enzyme proteins were according to previous reports.[31] Proteins (50 μg of protein/lane) were electrophoretically resolved using polyacrylamide gels (8, 10, or 12% resolving, 4% stacking) and transblotted overnight at 4 °C onto PVDF membrane (Millipore, Bedford, MA). The membranes were blocked with 2% nonfat dry milk (5% nonfat dry milk for hepatocyte nuclear factor 1α (HNF1α)) in PBS with 0.1% Tween (PBS/T) for 1 h and then incubated for 2 h with the primary antibody diluted in blocking buffer (PBS/T with 2% or 5% milk)) (all at room temperature). After washing, the membranes were incubated for 1 h with a species-appropriate peroxidase-labeled secondary antibody (Sigma-Aldrich, St. Louis, MO) diluted in 2 or 5% milk-PBS/T at room temperature. Table 1 describes the specific information and conditions used for the primary and secondary antibodies used in this study. After incubation with the secondary antibody, membranes were washed, incubated with ECL chemiluminescent kit (Amersham Life Science, Arlington Heights, IL), and exposed to Fuji medical X-ray film (Fisher Scientific, Springfield, NJ). The intensity of the protein bands was quantified using Kodak Molecular Imaging Software (Vision 4.0.4, Eastman Kodak Co.).

Membranes were stripped and reprobed for Mrp2, 6, Cyp3a11, and Cyp2e1 immunostaining and detection for liver

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Table 2. Liver, Kidney, and Body Weights, and Serum Glucose Levels in 11-Week-Old Wild-Type and Ob/Ob Mice

strain	gender	liver wt (g)	kidney wt (g)	average body wt (g)	liver/body wt	glucose levels (mg glucose/mL serum)
wild-type	female	0.87 ± 0.06	0.22 ± 0.03	20.6	0.04	1.58 ± 0.07
	male	1.14 ± 0.11[a]	0.32 ± 0.04[a]	27.2	0.04	1.57 ± 0.10
ob/ob	female	3.61 ± 0.64[b]	0.36 ± 0.03[b]	59.0	0.06	3.88 ± 0.14[b]
	male	4.49 ± 1.09[b]	0.43 ± 0.05*[b]	58.4	0.08	3.86 ± 0.02[b]

[a] Significant difference ($p \leq 0.05$) compared with corresponding female mice (same strain). [b] Significant difference ($p \leq 0.05$) compared to corresponding wild-type mice (same gender).

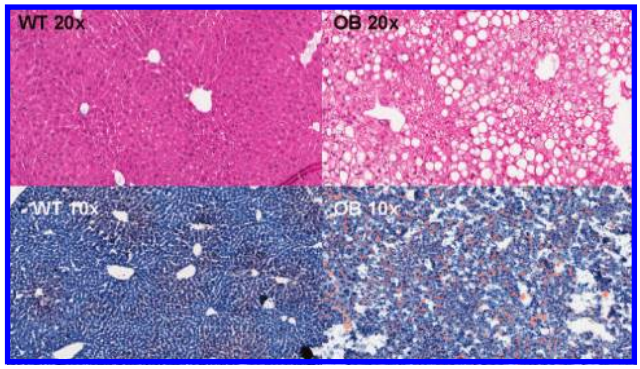

Figure 1. Hematoxylin and eosin (H&E) (top, magnification 20×) and Oil Red O staining (bottom, magnification 10×) of liver sections from wild-type and ob/ob mice. For H&E staining, liver tissues were stored in 75% ethanol after 24 h of fixation with 10% formaldehyde solution. After routine processing, livers were embedded in paraffin and sectioned to 5 μM and then stained with hematoxylin and eosin. For Oil Red O staining, frozen, unfixed livers were cryosectioned to 5–10 μM and then stained with Oil Red O.

samples. Equal protein loading was confirmed by coomassie stain of PVDF membranes.

Statistical Analysis. Statistical analyses of differences were performed by Student's *t* test. $P < 0.05$ was considered statistically significant. Unless otherwise stated, all data were presented as mean ± SE of five animals.

Results

Liver, Kidney, Body Weight, Serum Glucose Levels, And Hepatic Fat Accumulation in ob/ob Mice. Table 2 illustrates liver, kidney, and body weight, liver/body weight ratio, and serum glucose levels in ob/ob mice and wild-type controls at 11 weeks old. The liver weights of ob/ob mice were approximately 3-fold higher than that of the wild-type mice, and the ob/ob body weights were 1–2 fold higher than that of the wild-type mice. Serum glucose levels in ob/ob mice were more than twice of that in serum of wild-type mice, which indicated that these ob/ob mice had diabetes. Both hematoxylin and eosin staining and Oil Red O staining of liver sections showed accumulation of fat in livers from ob/ob mice (Figure 1). Histopathological observation of the wild-type liver appeared normal. Whereas in the ob/ob mice, microvasicular or monolocular vacuolations were observed in nearly all of the hepatocytes, as indicated by positive Oil Red O staining. No hepatic necrosis, inflammation or fibrosis was present in either strain, except for microgranuloma (a spontaneous lesion in rodents).

Liver Expression of Drug-Metabolizing Enzymes in ob/ob Mice. Figure 2 illustrates the mRNA expression of several DMEs in livers of ob/ob and wild-type mice. Some of these genes were selected because they are known to be induced through certain nuclear hormone receptor-mediated transcriptional pathways (i.e., Cyp4a14–PPARa, Cyp3a11–PXR, Cyp2b10 and Sult2a1/2–CAR, and Nqo1 and Ho-1–Nrf2). In livers of female ob/ob mice, Nqo1 mRNA expression increased by 40%, whereas Cyp2b10, 3a11, and 2e1 expression was decreased to around 60% of that in livers of wild-type females. Cyp4a14, Ho-1, and Sult2a1/2 mRNA expression in females did not differ significantly between ob/ob and wild types. In males, mRNA levels of Cyp4a14, 2b10, Ho-1, and Nqo1 in livers of ob/ob mice were increased 14.3, 2.3, 1.4, and 3.6-fold, respectively. In contrast, Cyp2e1 expression was decreased to 75% in livers of ob/ob mice as compared to that expressed in livers of wild-type males. Cyp3a11 mRNA expression was similar in livers of male ob/ob and wild-type mice. Significant gender differences were observed in livers of wild-type mice with Cyp4a, 2b10, 2e1, Nqo1, and Sult2a1/2 mRNA expression in females being 14.2-, 3.1-, 1.1-, 1.8-, and 130.9-fold higher, respectively, as compared to males. In ob/ob mice, Ho-1 and Sult2a1/2 mRNA expression was 1.4-fold lower and 3.4-fold higher in female livers, respectively, as compared to that in males.

Because of limited antibody availability, only Ho-1, Nqo1, Cyp2e1, and 3a11 protein expression was analyzed by Western blot (Figure 3). Consistent with the observed mRNA expression, Ho-1 protein expression was significantly decreased in livers of ob/ob female mice (63% of that in wild types), but 3.2-fold increased in livers of ob/ob male mice. Nqo1 protein expression was increased by 1.8 and 6.7-fold in livers of female and male ob/ob mice, respectively. In females, Cyp2e1 and 3a11 protein expression in liver did not differ between wild-type and ob/ob mice.

However, in males, Cyp2e1 and 3a11 protein expression in livers of ob/ob mice was significantly decreased to 82% and 58% of that in wild-types, respectively.

Liver Expression of Uptake Transporters in ob/ob Mice. Uptake solute carriers are localized to the basolateral membrane and transport xenobiotics, as well as other chemicals, into the hepatocytes. Figure 4 illustrates the mRNA expression of Oatp1a1, 1a4, 1b2, 1a6, 2b1, and Ntcp in livers of ob/ob and wild-type mice. In general, the mRNA expression of these transcripts was decreased in livers of both

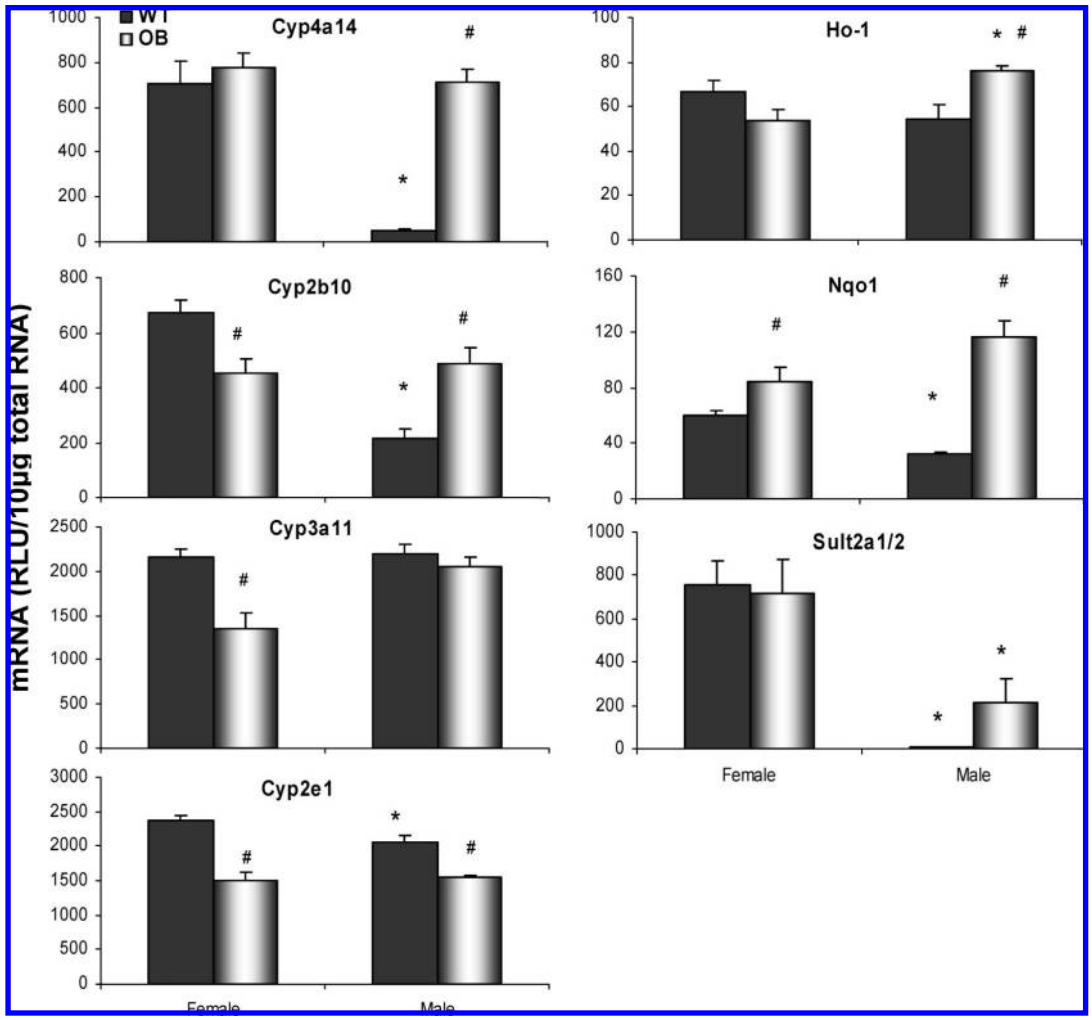

Figure 2. Drug-metabolizing enzymes (DMEs), cytochrome P450 (Cyp) 4a14, 2b10, 3a11, 2e1, heme oxygenase-1 (Ho-1), NAD(P)H:quinone oxidoreductase 1 (Nqo1), and sulfotransferase (Sult) 2a1/2 mRNA expression in livers of wild-type and ob/ob mice. Total RNA was isolated from liver, and mRNA levels were quantified using branched DNA signal amplification assay. The data is presented as mean RLU ± SEM ($n = 5$ animals). # represent a statistical difference between wild-type and ob/ob mice in the same gender ($p \leq 0.05$); asterisks (*) represent a statistical difference between female and male in the same strain ($p \leq 0.05$).

female and male ob/ob mice as compared to that in wild-types. In livers of ob/ob females, Oatp1a1 and 1a4 mRNA expression were decreased dramatically to 1% and 30% of that detected in livers of wild-type females, respectively. Oatp1b2 and Ntcp mRNA expression in livers of ob/ob females was also significantly decreased to about 72% of that in detected in livers of wild-type females. Oatp2b1 mRNA expression did not differ in livers of ob/ob and wild-type females. In males, Oatp1a1 mRNA expression in ob/ob livers was almost abolished and was 0.5% of that detected in livers of wild-type mice. Oatp1a4 expression in livers of male ob/ob mice was approximately half of that detected in livers of wild-type male mice. Oatp1b2, 2b1, and Ntcp mRNA expression did not differ in livers of ob/ob and wild type males. Significant gender differences were observed in livers of wild-type mice for Oatp1a1, 1a4, 2b1, and Ntcp, with 2.6-fold higher Oatp1 mRNA levels expressed in males, 1.8, 1.3, and 1.5-fold higher Oatp1a4, 2b1, and Ntcp mRNA levels in females, respectively.

Figure 5 illustrates relative protein expression of Oatp1a1, 1a4, b2, and Ntcp in livers of ob/ob as compared to wild-type mice. Consistent with the observed mRNA expression, a significant decrease in Oatp1a1 protein expression levels in livers of both female and male ob/ob mice was shown by Western blots; protein expression of Oatp1a1 in ob/ob females and males was 10% and 15% of that in wild-types, respectively. Protein expression of Oatp1a4 was 2.4 and 1.2-fold increased in ob/ob females and males, respectively; however, a significant decrease of Oatp1a4 mRNA expression levels was observed in ob/ob mice for both genders. Oatp1b2 protein expression was significantly increased by 130% in livers of ob/ob females and decreased in ob/ob males to 74% as compared to wild-types. However, Oatp1b2 mRNA expression was significantly decreased in livers of ob/ob females. Ntcp protein expression in ob/ob female and male mice was decreased to 66% and 49% of that in wild-types, respectively.

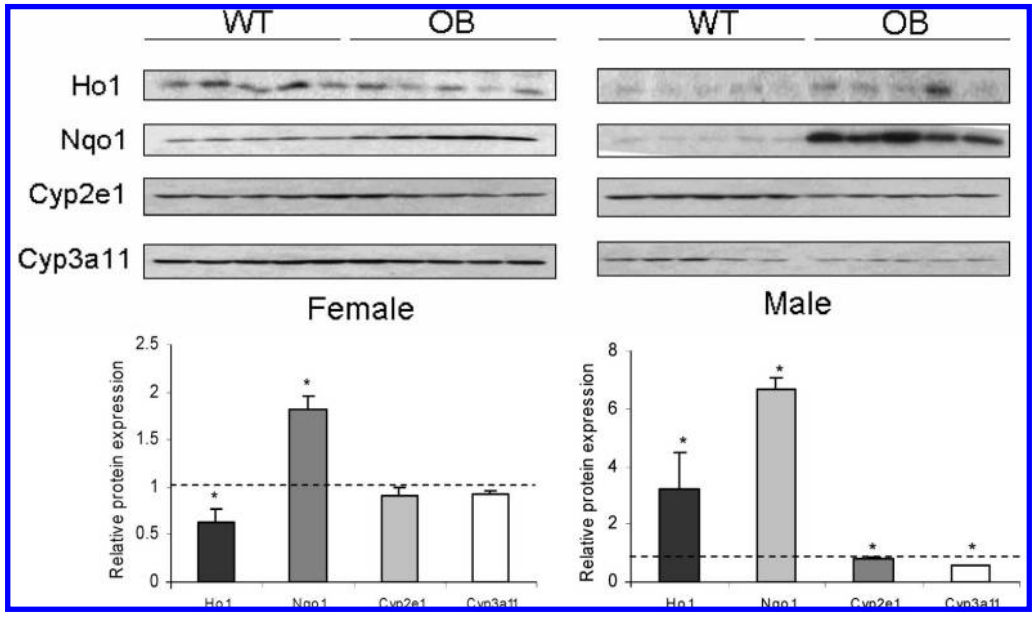

Figure 3. Western blot analysis of biotransformation enzymes heme oxygenase-1(Ho-1), NAD(P)H:quinone oxidoreductase 1 (Nqo1), cytochrome P450 (Cyp) 2e1, and 3a11 protein expression in livers from wild-type and ob/ob mice. Individual blots of female (left) and male (right) protein expression patterns are presented in the upper panels (protein loading amount 50 μg/lane, $n = 5$ animals). Quantification of relative protein expression in ob/ob mice compared to wild-types is illustrated in the bottom figures. Asterisks (*) represent a statistical difference ($p \leq 0.05$) from corresponding wild-type mice.

Liver Expression of Efflux Transporters in ob/ob Mice. Efflux transporters are localized to basolateral and canalicular membranes and function to transfer bile acids, drugs, and metabolites from hepatocytes to blood or bile, respectively. Among the Mrp family, Mrp1 and 3–6 are localized to basolateral membrane, whereas Mrp2 is localized to the canalicular membrane. Figure 6 illustrates the mRNA expression of Mrp1,3–6 in livers of ob/ob and wild-type mice. In livers of ob/ob females, Mrp1 and 6 mRNA expression levels were decreased to 58% and 62%, whereas Mrp4 mRNA expression was increased by 90% of that detected in wild-type females. Mrp3 and 5 mRNA expression in livers did not differ significantly between ob/ob and wild-type females. In ob/ob males, Mrp3–5 mRNA expression levels in liver increased 1.6-, 5.3-, and 1.5-fold, respectively, and no significant difference was observed for Mrp1 and 6 mRNA expression between ob/ob and wild-types. Significant gender difference was observed in wild types for Mrp3, 4, and 6 mRNA expression in livers, which was 1.4-, 2.0-, and 1.4-fold higher in females, respectively.

Consistent with observed mRNA expression, Western analyses showed a marked increase of Mrp4 protein expression level in ob/ob mice for both genders compared to wild-type; Mrp4 protein level in ob/ob females was 6.5-fold higher than in wild-type females, and for males, the protein was highly expressed in ob/ob while not detectable in wild-type (Figure 7). Mrp1 protein expression decreased significantly in ob/ob females (58% of that in wild-type females), while it was 3.2-fold higher in ob/ob males as compared to wild-types. Mrp3 and 5 protein expression was similar in livers of ob/ob and wild-type female mice. In contrast in males, Mrp3 and 5 protein expression was increased by 2.5- and 3.0-fold in livers of ob/ob mice, respectively, as compared to that in wild-types. Mrp6 protein expression in livers of ob/ob females and males decreased to 63% and 36% of that in wild-types, respectively.

Four canalicular efflux transporters, namely Mrp2, Mdr2, Bsep, and Bcrp, were analyzed for mRNA expression levels (Figure 8). In livers of ob/ob females, Bcrp mRNA expression was 1.6-fold increased; Bsep mRNA expression decreased to 59% of that in wild-types. No significant difference was observed for Mrp2 and Mdr2 between ob/ob and wild type females. In livers of ob/ob males, mRNA expression of Mdr2 and Bcrp was increased by 1.4- and 1.2-fold, respectively, while mRNA expression of Mrp2 and Bsep was similar between ob/ob and wild types. Significant gender difference was observed for Mdr2 and Bcrp mRNA expression in livers of ob/ob mice, being 1.4- and 1.5-fold higher in males than females, respectively. Bcrp mRNA expression in livers also showed gender difference in wild-types with 2-fold higher in males than in females.

Because of the unavailability of antibodies for Mdr2 and Bsep, only Bcrp and Mrp2 protein expression was analyzed by Western blot (Figure 9). Bcrp protein expression level increased significantly by 2.2-fold in ob/ob females, and Mrp2 protein expression levels in ob/ob mice were 3.4- and 2.9-fold higher in females and males, respectively, as compared to that in wild types.

Kidney Oatp1a1, Oat2, Mrp3, 4, and Nqo1 mRNA and Protein Expression in ob/ob Mice. Oatp 1a1, 1a4, 1a6, Oat1–3, Oct1–3, Mrp1–4, Bcrp, and Nqo1 mRNA expression

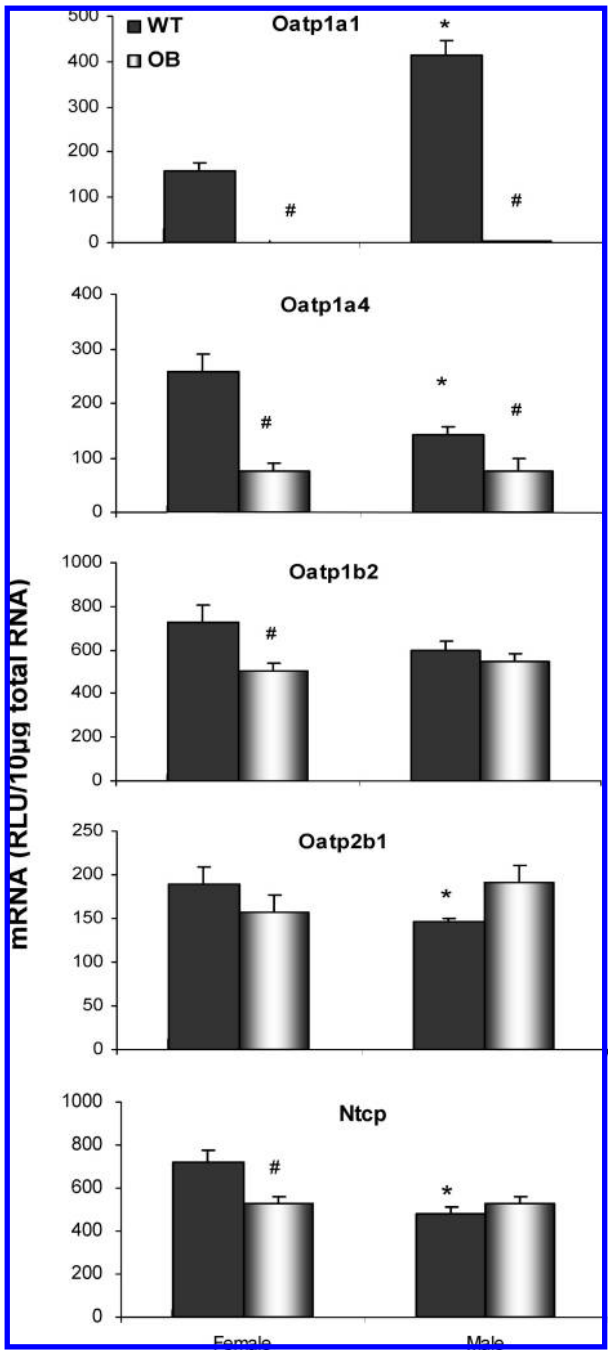

Figure 4. Uptake transporters organic anion-transporting polypeptide (Oatp) 1a1, 1a4, 1b2, 2b1 and sodium/taurocholate cotransporting polypeptide (Ntcp) mRNA expression levels in livers of ob/ob mice. Total RNA was isolated from liver, and mRNA levels were quantified using branched DNA. The data is presented as mean RLU ± SEM ($n = 5$ animals). # represent a statistical difference between wild-type and ob/ob mice in the same gender ($p \leq 0.05$); asterisks (*) represent a statistical difference between female and male in the same strain ($p \leq 0.05$).

was analyzed in kidneys of ob/ob and wild-type mice, but only data for Oatp1a1, Oat2, Mrp3–4, and Nqo1 expression is presented in this manuscript (Figure 10A) because marked expression differences between ob/ob and wild-type controls were observed for those transcripts. In ob/ob females, mRNA levels of Oatp1a1, Oat2, and Mrp3 in kidney were markedly decreased to 4%, 5%, and 18% of that detected in wild-types, respectively, and Mrp4 and Nqo1 mRNA expression increased 90% and 170%, respectively. In kidneys of ob/ob males, Oatp1a1 and Oat2 mRNA expression was decreased to 16% and 4% of that in wild-types, respectively, and Mrp4 and Nqo1 mRNA expression was increased by 260 and 90%, respectively. Mrp3 mRNA expression in kidneys of males was very low in both ob/ob and wild-types and did not differ significantly between these two strains. Additionally, gender-divergent Oatp1a1, Mrp3, 4, and Nqo1 expression in kidney was observed in wild-types, with Oatp1a1 and Nqo1 levels being 8- and 1.3-fold higher in males, respectively, Mrp3 and 4 levels being 80 and 2-fold higher in females, respectively. Significant gender difference was also observed for Mrp3 mRNA expression in kidneys of ob/ob mice with 18-fold higher in females.

Western analysis demonstrated that Mrp3 protein expression in kidneys of female ob/ob mice was also dramatically decreased to 12% of that in wild-types (Figure 10B). Gender-divergent expression of Mrp3 protein in kidneys was observed in wild-types, with no detectable protein expression in males and pretty high expression in females. Mrp4 protein expression was significantly higher in kidneys of both female and male ob/ob mice than wild-type controls, with 2.6- and 5.0-fold increased, respectively (Figure 10B).

HNF1α Protein Expression in Liver Nuclear Fractions of Wild-Type and ob/ob Mice. Considering hepatocyte nuclear factor 1α (HNF1α) regulates several hepatic transporters expression, such as Oatp1a1,[32] its protein expression was analyzed by Western blot, and the results are shown in Figure 11. HNF1a protein expression was determined in nuclear fractions isolated from livers of wild-type and ob/ob mice. HNF1α protein expression in nuclear fractions from livers of ob/ob female and male mice was significantly decreased to 35% and 37% of that detected in nuclear fractions isolated from livers of wild-type mice, respectively.

Discussion

The current study demonstrates that ob/ob mice, which model obesity, diabetes, and fatty liver, exhibit altered expression of important DMEs and transporters in liver and kidney. Because some DMEs and transporters are regulated similarly for mice and humans, data from this study suggest that ob/ob mice may be a useful tool to predict/model drug pharmacokinetics in obese/diabetic humans. Additionally, the expression of certain DMEs and transporters was analyzed

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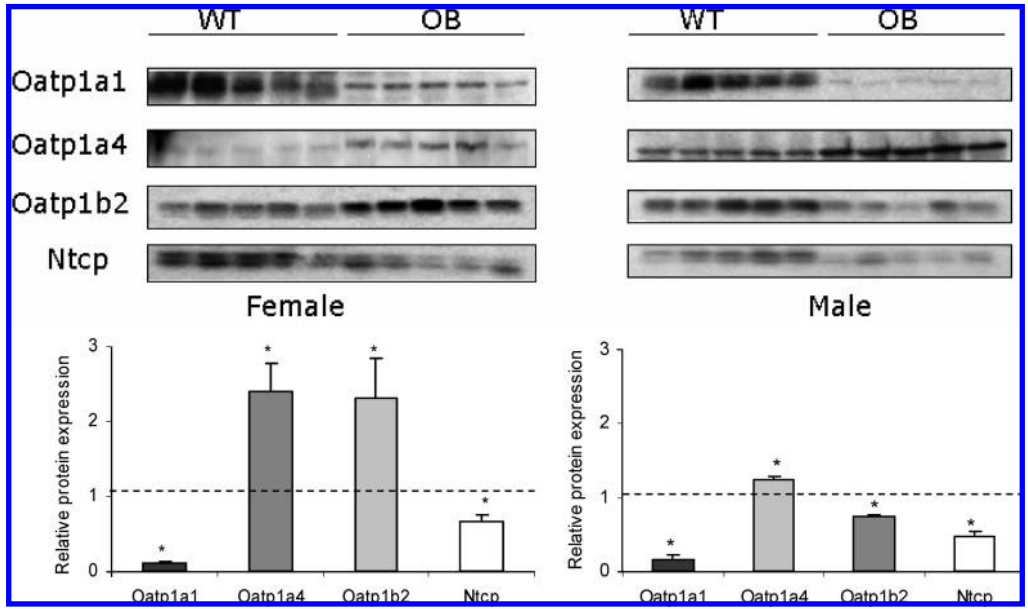

Figure 5. Western blot analysis of sodium/taurocholate cotransporting polypeptide (Ntcp), organic anion-transporting polypeptide (Oatp) 1a1, 1a4, and 1b2 protein expression in livers of wild-type and ob/ob mice. Individual blots are presented on the top (protein loading amount 50 μg/lane, $n = 5$ animals). Quantification of relative protein expression in ob/ob mice compared to wild-types is illustrated in the bottom figures. Asterisks (*) represent a statistical difference ($p \leq 0.05$) from corresponding wild-type mice.

to assess activation of specific nuclear hormone receptor mediated pathways. Overall, data in this manuscript suggests that PPARα, CAR, and Nrf2 may be activated, whereas HNF1a expression is decreased in liver during obesity and diabetes.

Cyp2e1 has been well documented to be increased in the liver of obese and diabetic humans and some animal models.[16,33] However, some previous reports also demonstrated unchanged or decreased Cyp2e1 mRNA and/or protein expression in livers of ob/ob mice, obese Zucker rats, or db/db (diabetic) mice.[34–36] In the current study, significant reduction of Cyp2e1 mRNA expression was observed in livers of both female and male ob/ob mice, and the protein expression level in liver was similar between female ob/ob and wild-type mice. The ob/ob mice used in current study have a mutation in the leptin gene, and it was proposed that the absence of leptin or its receptor could account for the lack of increase of Cyp2e1 at either the transcriptional or post-transcriptional level in these genetic animal models of obesity and diabetes.[35] In addition to leptin, other hormones such as insulin also have been implicated in Cyp2e1 regulation, with reversing the increase of Cyp2e1 activity in livers of streptozotocin-induced diabetic rats and down-regulating Cyp2e1 expression in rat hepatoma cell line and primary cultured rat hepatocytes.[37–39] Thus, unchanged or decreased Cyp2e1 levels in these ob/ob mice were not completely unexpected, since these obese mice were hyperinsulinemic at all ages.[40] Additionally, Cyp2e1 has been characterized as ethanol-inducible and is associated with the pathogenic processes leading to alcoholic liver disease as well as nonalcoholic steatohepatitis (NASH).[41–43] Unchanged Cyp2e1 protein expression in livers of these ob/ob mice was also confirmed by histological analysis in which fat accumulation, not NASH, was observed in livers of the ob/ob mice.

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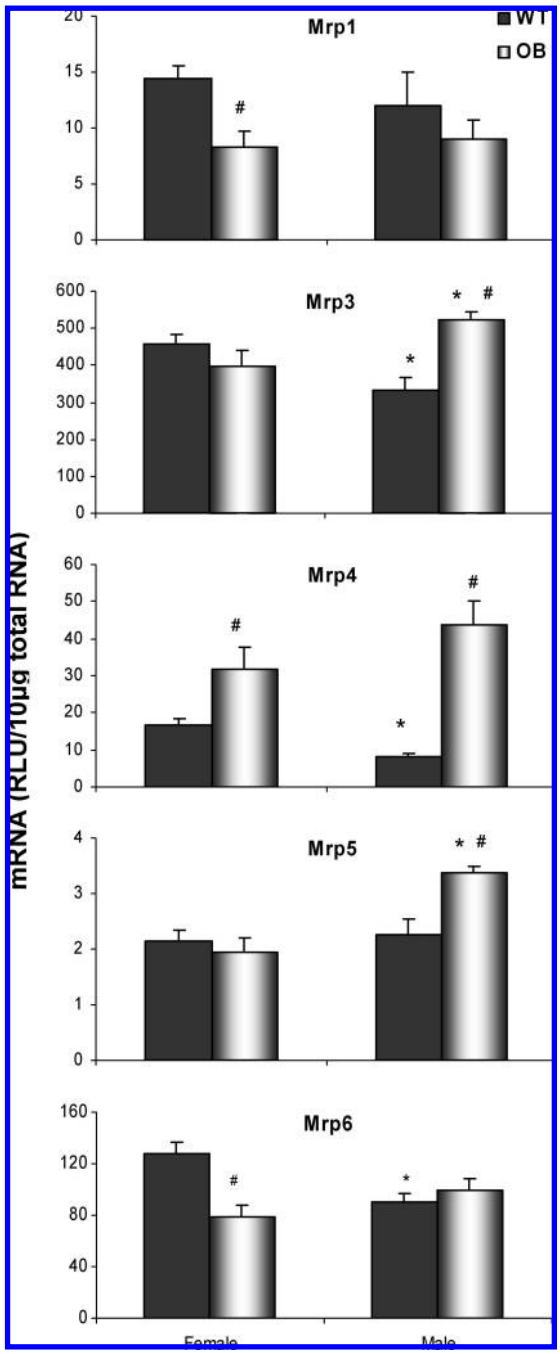

Figure 6. Multidrug resistance-associated protein (Mrp) 1, 3, 4, 5, and 6 mRNA expression in livers of wild-type and ob/ob mice. Total RNA was isolated from liver, and mRNA levels were quantified using branched DNA assay. The data is presented as mean RLU ± SEM (n = 5 animals). # represent a statistical difference between wild-type and ob/ob mice in the same gender ($p \leq 0.05$); asterisks (*) represent a statistical difference between female and male in the same strain ($p \leq 0.05$).

Increased Cyp4a14 mRNA expression, particularly in male ob/ob mice, was consistent with previous reports in which expression of Cyp4a10 and 4a14 in the obese mice and Cyp4as in diabetic mice (db/db mice) were greatly increased.[35,36] Cyp4a is important in the metabolism of fatty acids, and induction of Cyp4a expression has been shown to result from transcriptional activation mediated by peroxisome proliferator activated receptor (PPAR), which has been suggested to be activated by fatty acids.[44] In addition, Cyp4a mRNA has been suggested to be induced by type I diabetes, and an increase of Cyp4a protein levels and its specific enzymatic activity has been detected in the livers of diabetic rats treated with streptozotocin.[45,46] Although obesity-associated diabetes is different from diabetes induced by streptozotocin regarding their causes and symptoms, it does not exclude that Cyp4a could be induced by obesity-associated diabetes.

Decreased Cyp2B2 mRNA levels were observed in livers of Zucker obese rat as compared to the lean ones.[47] In contrast, increased Cyp2b10 mRNA was observed in livers of db/db mice,[36] which was consistent with the result in the current study: Cyp2b10 mRNA expression increased in livers of ob/ob male mice. Strain- and gender-selectivity has been documented in rodents regarding the induction of Cyp2b genes by phenobarbital;[48,49] therefore, species and gender differences could exist in terms of Cyp2b gene expression with regard to obesity, diabetes, and fatty liver disease. Phenobarbital-like compounds induce Cyp2b10 gene expres-

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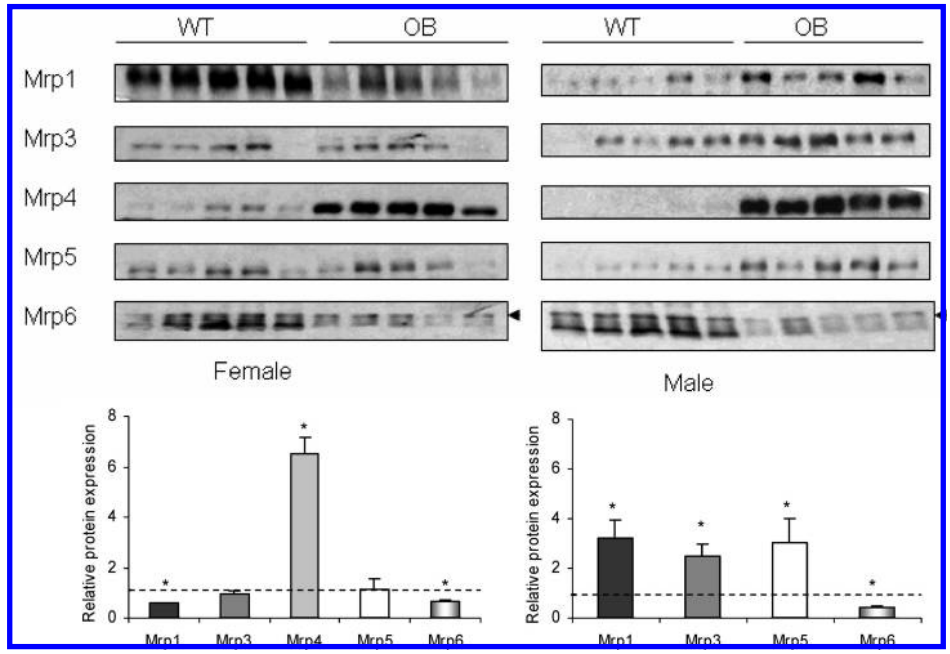

Figure 7. Western blot analysis of multidrug resistance-associated protein (Mrp) 1, 3, 4, 5, and 6 proteins expression in livers from ob/ob and corresponding wild-type mice. Individual blots are presented on the top (protein loading amount 50 μg/lane, $n = 5$ animals). Quantification of relative protein expression in livers of ob/ob mice compared to wild-types is illustrated in the bottom figures. Mrp4 was not quantified because levels in livers of wild-type males were undetectable. Arrows point Mrp6 bands. Asterisks (*) represent a statistical difference ($p \leq 0.05$) from corresponding wild-type mice.

sion through activation and nuclear translocation of the constitutive androstane receptor (CAR).[50] Thus, the observed increase of Cyp2b10 mRNA expression in ob/ob male mice suggests that CAR may be activated in livers with obesity, diabetes, and/or fatty liver. In addition to Cyp2b10, several other CAR targets genes such as Sult2a1 and Oatp1a4 were also induced in ob/ob mice, which further suggests CAR may be activated. CAR activation and induction of CAR target genes (Sult2a1, Ugt1a1, and Oatp1a4) during fasting were demonstrated to contribute to the decrease in basal metabolic rate,[51,52] which may also play a role in modulating weight loss and energy homeostasis in mammals with obesity and its associated metabolic disorders. During fasting, induced cAMP, PPARγ coactivator 1α (PGC-1α), and hepatocyte nuclear factor 4α (HNF4α) are correlated with CAR activation and induction of CAR target genes in liver. The network of interaction involving cAMP, PGC-1α, HNF4α, CAR, and its target genes could be mediated in obesity and diabetes as well.

Cellular defensive enzymes such as Ho-1 and Nqo1 also increased at both mRNA and protein levels in livers of ob/ob mice, particularly in males, which could be a protective response to obesity and fatty liver. Elevated Nqo1 mRNA expression was also observed in kidney of ob/ob mice in the current study. Transcription of Ho-1 and Nqo1 is mediated, in part, through activation of NF-E2-related factor-2 (Nrf2).[53,54] More recently, it has been shown that liver mRNA expression of mouse Mrp2–6 is induced by compounds that activate the Nrf2 transcriptional pathway.[55] Thus, the marked induction of Nqo1 and Mrp4 in current study may indicate that the Nrf2 transcriptional pathway is activated in livers and kidneys with obesity and diabetes.

Oatp1a1 was highly expressed in livers and kidneys of wild-type mice, especially in males, but was significantly lower in both liver and kidney of ob/ob mice. Oatp1a1 can mediate the transport of a wide range of amphipathic organic

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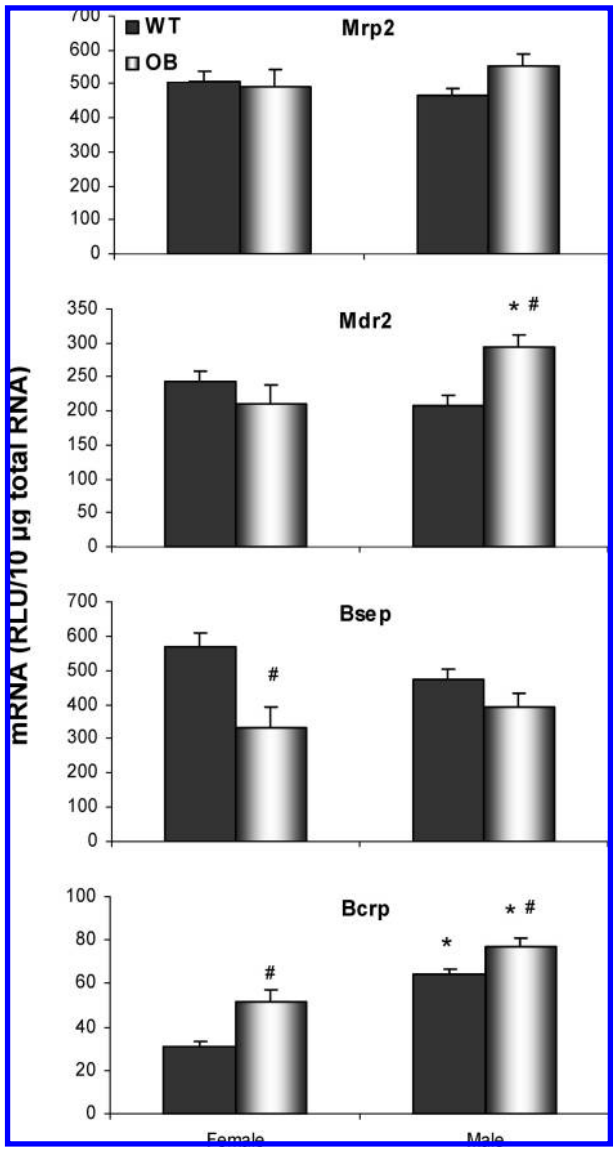

Figure 8. Multidrug resistance-associated protein 2 (Mrp2); multidrug resistance protein 2 (Mdr2); bile salt export pump (Bsep); breast cancer resistance protein (Bcrp) mRNA expression in livers of ob/ob and wild-type mice liver. Total RNA was isolated from liver, and mRNA levels were quantified using branched DNA assay. The data is presented as mean RLU ± SEM ($n = 5$ animals). # represent a statistical difference between wild-type and ob/ob mice in the same gender ($p \leq 0.05$); asterisks (*) represent a statistical difference between female and male in the same strain ($p \leq 0.05$).

compounds and numerous drugs.[56,57] Therefore, the dramatic decrease of Oatp1a1 in ob/ob mice liver and kidney could result in altered drug absorption and excretion and thus significantly affect drug pharmacokinetics and toxicity. Oatp1a4 also showed significant lower mRNA expression levels in ob/ob mice, which was consistent with previous reports in which a significant reduction of Oatp1a4 gene expression was observed in obese Zucker rats.[58] Although there are no human homologues for mouse Oatp1a1 and 1a4, human OATP-1B3 and 1B1, expressed in liver, have similar substrates, and their basal expression is regulated by similar transcriptional mechanisms. Therefore, it is important to examine OATP1B1 and 1B3 expression in livers of obese patients.

Expression of several hepatocyte transporters, namely Ntcp, Oatp1a1, 1a4, 1b2, Mdr2, 1b, and Bsep, has been reported to be regulated by hepatocyte nuclear factor 1α (HNF1α) in cholestatic and hepatic injury models.[59–64] Down-regulation of the basolateral organic transporters may occur by decreased HNF1 binding activity to regulatory elements present in promoters that maintain basal gene expression. For example, Oatp1a1 mRNA expression was markedly lower in liver and kidney of HNF1α-null mice than wild-types.[32] Analysis of the mouse Oatp1a1 promoter reveals five potential HNF1 binding sites located within the first 6000 base pairs. The promoters for human OATP1B3 and 1B1 contain HNF1α binding sequences, and targeted mutagenesis of the binding sites abolished baseline promoter

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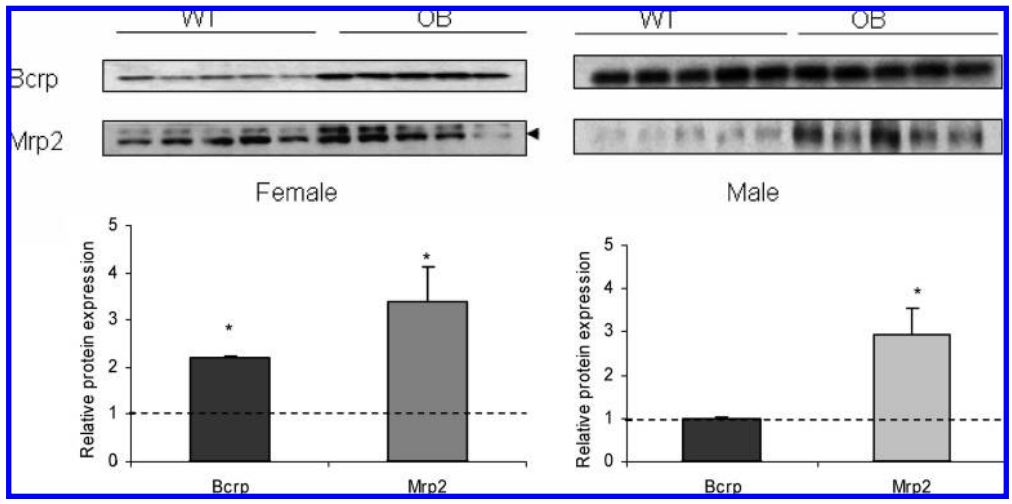

Figure 9. Western blot analysis of breast cancer resistance protein (Bcrp) and multidrug resistance-associated protein 2 (Mrp2) protein expression in livers from ob/ob and corresponding wild-type mice. Individual blots are presented on the top (protein loading amount 50 μg/lane, $n = 5$ animals). Quantification of relative protein expression in ob/ob mice compared to wild-types is illustrated in the bottom figures. Arrows point to Mrp2 bands. Asterisks (*) represent a statistical difference ($p \leq 0.05$) from corresponding wild-type mice.

activity in transfected hepatoma cell lines.[65] In the current study, decreased HNF1α protein expression was observed in nuclear fractions of livers from ob/ob mice, which indicates that decreased HNF1α expression and binding could be involved in the down regulation of Oatp1a1 in obesity, diabetes, and/or fatty liver disease.

Mrp4 (MRP4) transports bile acids, as well as many antiviral drugs, from hepatocytes into blood.[66,67] In human, MRP4 is also important for renal elimination of antiviral drugs and chemicals.[68,69] Increased Mrp4 mRNA and protein expression was reported in acute chemical-induced liver injury and extrohepatic obstructive cholestasis.[24,31,70,71] Studies have demonstrated that induction of mouse and human Mrp4/MRP4 is likely mediated by transcriptional activation of CAR and Nrf2.[55,72,73] In addition, PPARα-dependent activation was also reported in Mrp3 and 4 up-regulation by clofibrate.[74] In this study, Mrp4 mRNA and protein expression levels were increased in ob/ob mice liver and kidney. Although no data exist yet with regard to Mrp expression and obesity and its associated metabolic disease clusters, the significant change of Mrp4 in ob/ob mice liver and kidney in current study indicates disposition of toxicants, xenobiotics, and drugs that are substrates of Mrp4 may be different in obese and/or diabetic humans.

In general, uptake transporters including Oatps and Ntcp were decreased in livers from ob/ob mice at both the mRNA and protein expression levels. Conversely, the mRNA and protein expression of several Mrps localized to the basolateral membrane, namely Mrp3, 4, and 5, was increased in livers of male in ob/ob mice. In particular, Mrp4 protein expression

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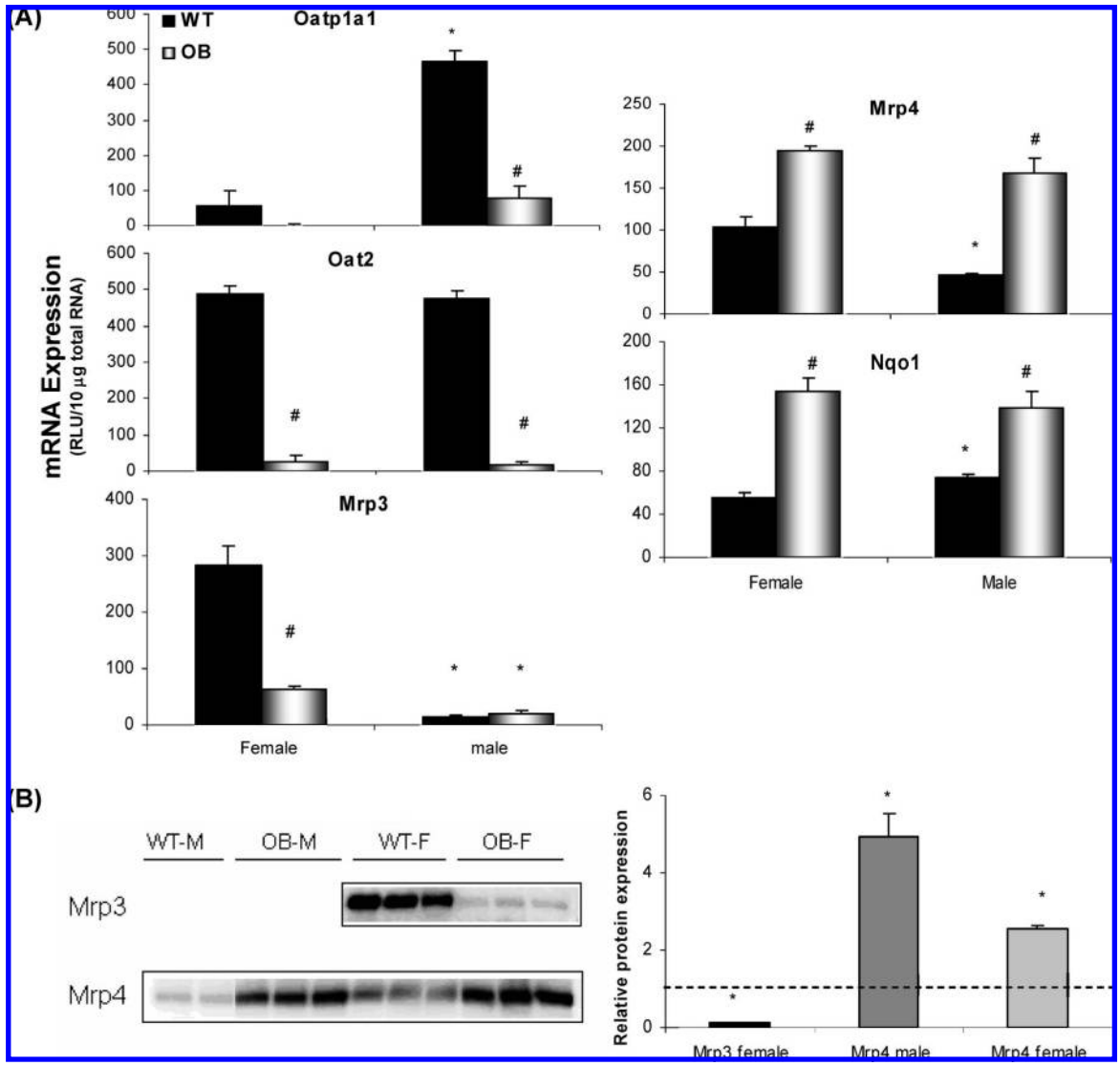

Figure 10. Organic anion-transporting polypeptide (Oatp) 1a1, organic anion transporter (Oat) 2, multidrug resistance-associated protein (Mrp) 3, 4, and NAD(P)H:quinone oxidoreductase 1 (Nqo1) mRNA and protein expression in ob/ob and wild-type mice kidney. (A) Oatp1, Oat2, Mrp3, Mrp4, and Nqo1 mRNA expression in kidneys of ob/ob and wild-type mice. Total RNA was isolated from liver and mRNA levels were quantified using branched DNA assay. The data is presented as mean RLU ± SEM ($n = 5$ animals). # represent a statistical difference between wild-type and ob/ob mice in the same gender ($p \leq 0.05$); asterisks (*) represent a statistical difference between female and male in the same strain ($p \leq 0.05$). (B) Western blot analysis of Mrp3 and 4 protein expression in kidneys from ob/ob and corresponding wild-type mice. Individual blots are presented in the left panel (protein loading amount 50 μg/lane, $n =$ 2 for wild-type male group (WT-M), and $n = 3$ animals for other three groups, ob/ob male (OB-M), wild-type female (WT-F), and ob/ob female (OB-F)). Quantification of Mrp4 relative protein expressions in ob/ob mice compared to wild-types is illustrated in the right panel. No quantification for Mrp3 in males because it was not detected in the kidneys of both wild-type and ob/ob males. Asterisks (*) represent a statistical difference ($p < 0.05$) from corresponding wild-type mice.

in liver was significantly increased in ob/ob mice for both genders. With extrapolation to humans, patients presenting obesity and/or diabetes may have decreased hepatic uptake and increased sinusoidal efflux. As a result, some drugs such as statins, which require hepatic metabolism and biliary excretion for elimination,[75] could have higher bioavailability and a longer half-life in the body and thus may lead to drug levels approaching a toxic threshold if parent drugs are more toxic. On the other hand, elevated expression levels of several DMEs in livers of male ob/ob mice, including Cyp4a, 2b10, Nqo1, and Sult2a1/2, suggested that detoxification of certain

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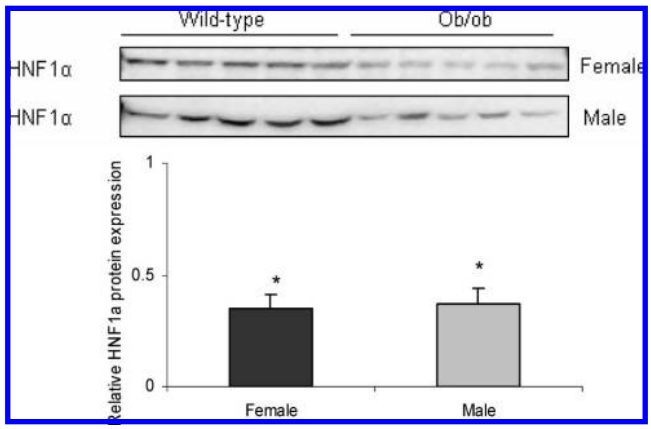

Figure 11. Western blot analysis of HNF1α protein expression in nuclear fractions from livers of wild-type and ob/ob mice. Individual blots are presented on the top and quantification of relative protein expression in ob/ob mice as compared to wild-types is illustrated in the bottom (protein loading amount 50 μg/lane, $n = 5$ animals). Asterisk (*) represent a statistical difference ($p < 0.05$) from corresponding wild-type mice.

drugs and xenobiotics could be increased in ob/ob patients and thus reduce the toxicity of chemical insult.

Several drugs being used to treat diabetes have been reported to cause severe adverse drug reactions, such as troglitazone, which was withdrawn from the market for fetal liver toxicity, and rosiglitazone, which was recently found to be associated with increased heart attacks.[76] With decreased elimination and active retention, these drugs may result in severe toxicity to those obese and/or diabetic patients. In addition, activation/suppression of certain nuclear transcription factors such as CAR, PPARα, Nrf2, and HNF1α in obesity and diabetes could also activate/suppress their target genes in other no-target organs such as heart, in addition to liver, resulting complex biological effects. Accordingly, many different and some seemingly unrelated toxic effects of these antidiabetic drugs could emerge in morbidly obese and/or diabetic humans.

Gender-divergent mRNA expression was observed for several DMEs and hepatic transporters in livers of wild-type mice, particularly for Cyp4a, Sult2a1/2, and Oatp1, which could result in gender-related pharmacokinetics and toxicity of certain drugs that are substrates of these enzymes and transporters in obese humans. In addition, a different change pattern of several DMEs and transporters mRNA expression was also showed in ob/ob mice compared to wild-type between female and male, such as significant increase of Cyp2b10, Ho-1, Mrp3, and Mdr2 mRNA in ob/ob male, while decrease in ob/ob female. Hormonal regulation or signaling would play a role in the different response to deletion of leptin gene between females and males, as the actions of many steroid hormones on metabolism are often sex-specific. Same hormone could have opposite impact on obesity and diabetes between women and men; for example, high testosterone levels are associated with higher risk of type II diabetes in women, but with lower risk in men. Links exist between estrogen and the regulation obesity, and thus, menopause makes women fat.

In summary, mRNA and/or protein expression levels of several DMEs and hepatic transporters were different in livers and kidneys of ob/ob mice than wild-types, which suggest it is possible for changed pharmacokinetics and drug−drug interactions to occur in obese and/or diabetic humans. In the future, functional assays will be conducted to examine activity of those DMEs and transporters with altered expression levels. Activation of certain nuclear hormone receptors, such as CAR, Nrf2, and PPARα, should be further examined to confirm their regulation on some DMEs with changed expression levels and, thus, potential mechanisms involved in obesity and associated diabetes. In addition, on the basis of the fundamental data, future disposition studies will be carried to examine the disposition of some prototypical compounds and commonly prescribed classes of drugs such as diabetes and antihypolipidenia drugs. Overall, this study provides novel new information regarding the regulation of DMEs and transporters by certain nuclear hormone receptors in liver and kidney in a mouse model of obesity and diabetes. The useful information will aid further study of DME and transporter expression in humans diagnosed with obesity and/or diabetes and provide a possible explanation of the differences in drug pharmacokinetics and toxicity in morbidly obese humans.

Abbreviations Used

Bcrp, breast cancer resistance protein; bDNA, branched DNA signal amplification; Bsep, bile salt-export pump; Cyp, Cytochrome P450; DMEs, drug metabolizing enzymes; Gclc, glutamate-L-cysteine ligase catalytic subunit; HNF1α, hepatocyte nuclear factor 1α; Ho-1, heme oxygenase-1; Mdr2, multidrug resistance protein 2; Mrp, multidrug resistance-associated protein; Nqo1, NAD(P)H:quinone oxidoreductase 1; Nrf2, nuclear factor-E2-related factor 2; Ntcp, sodium/taurocholate cotransporting polypeptide; Oat, organic anion transporter; Oatp, organic anion transporting polypeptide; Oct, organic cation transporter; Sult2a1/2, sulfotransferase 2a1/2.

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