Impact of Hyperlipidemia on Plasma Protein Binding and Hepatic Drug Transporter and Metabolic Enzyme Regulation in a Rat Model of Gestational Diabetes

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ABSTRACT

It is currently unknown whether gestational diabetes mellitus (GDM), a prevalent obstetrical complication, compounds the changes in drug disposition that occur naturally in pregnancy. Hyperlipidemia occurs in GDM. Using a rat model of GDM, we determined whether excess lipids compete with drugs for plasma protein binding. Because lipids activate nuclear receptors that regulate drug transporters and metabolic enzymes, we used proteome analysis to determine whether hyperlipidemia indirectly leads to the dysregulation of these proteins in the liver. GDM was induced on gestational day 6 (GD6) via streptozotocin injection. Controls received either vehicle alone or streptozotocin with subsequent insulin treatment. Liver and plasma were collected on GD20. Glyburide and saquinavir protein binding was determined by ultrafiltration, and an established solvent method was used for plasma delipidation. Proteomics analysis was performed by using isobaric tags for relative and absolute quantitation methodology with membrane-enriched hepatic protein samples. Relative to controls, GDM rat plasma contained more cholesterol and triglycerides. Plasma protein binding of glyburide and saquinavir was decreased in GDM. Delipidation normalized protein binding in GDM plasma. Proteins linked to lipid metabolism were strongly affected in the GDM proteomics data set, with prohyperlipidemic and antihyperlipidemic changes observed, and formed networks that implicated several nuclear receptors. Up-regulation of drug transporters and metabolic enzymes was observed (e.g., multidrug resistance 1/2, CYP2A1, CYP2B9, and CYP2D3). In this study, GDM-induced hyperlipidemia decreased protein binding and was associated with drug transporter and metabolic enzyme up-regulation in the liver. Both of these findings could change drug disposition in affected pregnancies, compounding changes associated with pregnancy itself.

Gestational diabetes mellitus (GDM) is an obstetrical complication that affects up to 8% of all pregnancies in North America (Lawrence et al., 2008). At present, little is known about how the mechanisms that determine drug disposition differ in women with this condition relative to women with uncomplicated pregnancies. Although it has been established that pregnancy itself decreases the plasma protein binding of drugs (Perucca et al., 1981; Notarianni, 1990; Tseng et al., 1999), there have been few attempts to determine whether maternal diseases such as GDM alter this parameter further. Given the difficulty associated with dosage determination in pregnancy, the potential compounding effects of maternal disease on protein binding could represent a largely overlooked source of variability.

Protein binding strongly influences a drug’s distribution and/or clearance. With distribution, placental transfer is limited to unbound drugs, so altered protein binding in pregnancy can significantly affect the materno-fetal transfer of
plasma proteins such as albumin and studies have implicated increased glycosylation of abundant than in healthy volunteers (Arredondo et al., 1999). Some patients with type 1 and type 2 diabetes is significantly less cause there are reports of changes to this parameter in other (when increased) or toxicity (when decreased).

Protein binding in GDM is of interest not just because there is a paucity of information concerned with it but because there are reports of changes to this parameter in other forms of diabetes (Ruiz-Cabello and Erill, 1984; Trovik et al., 1992; Arredondo et al., 1999). For example, it has been demonstrated that the plasma protein binding of itraconazole in patients with type 1 and type 2 diabetes is significantly less than in healthy volunteers (Arredondo et al., 1999). Some studies have implicated increased glycosylation of abundant plasma proteins such as albumin and α1-acid glycoprotein (AAG) (Wörner et al., 1992; Koyama et al., 1997), but reports on this topic are conflicting (Olsen et al., 1995).

Hyperlipidemia is a common comorbidity among patients with type 1 or 2 diabetes (Ginsberg, 1996; Betteridge, 2000; Krentz, 2003; Harris et al., 2005) and represents an alternative explanation for altered protein binding in diabetes (Ruiz-Cabello and Erill, 1984; de la Fuente et al., 2002). In a study using patients with type 1 diabetes, a significant positive correlation between elevated plasma lipids, such as cholesterol and triglycerides, and the urinary excretion of albumin was observed (Mattock et al., 2001). In addition to increasing the urinary excretion of this important plasma protein, it is known that lipids can interact directly with proteins to alter their capacity for drug binding via competition or allosteric modulation. Even small increases in free fatty acids (FFAs) displace chlorophenoxyisobutyrate from human albumin (Spector et al., 1973). Along these same lines, the binding of propranolol to human AAG can be significantly increased if AAG-bound lipids are removed first (Chauvelot-Moachon et al., 1988). In pregnancy, diabetes exacerbates the natural rise in plasma lipids that occurs across pregnancy (Couch et al., 1998; Basaran, 2009).

In this study, plasma lipids and protein binding were examined in the streptozotocin (STZ) rat model of GDM. Similar to the clinical scenario, STZ-induced diabetes is associated with hyperlipidemia (Wasan et al., 1998), and our initial experiments with nonpregnant rats have demonstrated correlations between plasma lipids and the unbound percentage of both acidic and basic drugs after STZ injection (unpublished data). We hypothesized that excess plasma lipids would compete with drugs for protein binding in rats with STZ-induced GDM. In addition to assessing plasma lipids and protein binding, insight into the effects of hyperlipidemia on hepatic proteins linked to drug transport and metabolism was sought by using a global proteomics approach (8Plex iTRAQ). The regulation of drug transporters and metabolic enzymes in the liver is controlled largely by nuclear receptors, such as the farnesoid X receptor (FXR) and pregene X receptor (PXR), which are themselves activated by various plasma lipids (Handschin and Meyer, 2005). Dysregulation of hepatic drug transporters and metabolic enzymes would be expected to exacerbate any variability in drug disposition introduced by altered plasma protein binding. We hypothesized that hyperlipidemia in STZ-induced GDM would indirectly result in hepatic drug transporter and metabolic enzyme dysregulation.

Our findings advance our understanding of the impact that GDM-induced hyperlipidemia can have on plasma protein binding and the expression of drug transporters and metabolic enzymes in the liver.

Materials and Methods

Animals. Pregnant Sprague-Dawley rats (207–260 g) were purchased from Charles River Laboratories, Inc. (Seneville, QC, Canada) and housed individually in a temperature-controlled facility on a 12:12-h light/dark cycle. Rats were given free access to water and standard chow [Harlan Teklad (Madison, WI) Global Diet 2018]. Experiments were approved by the Office of Research Ethics at the University of Toronto and performed in accordance with Canadian Council on Animal Care Guidelines.

Animal Treatments/Monitoring and Tissue Collection. Rats were randomly assigned to one of three groups (n = 5/group): non-treated diabetics (the GDM group), vehicle controls (the vehicle group), and insulin-treated diabetics (the insulin-treated group). Animals in the vehicle and insulin-treated groups are collectively referred to as controls. On gestational day 6 (GD6), diabetes was induced by way of a single subcutaneous injection of STZ (PubChem Compound ID 5300; Sigma-Aldrich, Oakville, ON, Canada) in 0.1 M citrate buffer (pH 4.5) at a dosage of 45 mg/kg. Vehicle control animals received a single subcutaneous injection of citrate buffer on GD6. For all animals, body weight was measured before injections (GD6) and then daily until sacrifice. For animals in the GDM and vehicle groups, blood glucose was measured before injections (GD6) and on GDs 7, 8, 9, 11, 13, 15, 18, and 20. For animals in the insulin-treated group, blood glucose was measured before STZ injection (GD6) and then daily until sacrifice to determine daily insulin dosage requirements. Blood glucose was measured at noon, in a nonfasted state, by using a commercially available glucometer (Free-style Freedom Meter, Abbott Laboratories, Alameda, CA) with blood obtained via tail prick. When values exceeded the range of this glucometer, a commercially available enzymatic glucose assay was used according to the manufacturer’s instructions (Autokit Glucose; Wako Chemicals USA, Inc., Richmond, VA). Mild hyperglycemia was defined as blood glucose concentration at 10 to 14 mM, and diabetes was defined as blood glucose concentration at >14 mM. For diabetic rats receiving insulin treatment, treatment was initiated once diabetes was confirmed and consisted of an initial subcutaneous injection of 8 to 10 U/kg Humulin-L (human biosynthetic intermediate-insulin; Eli Lilly Canada Inc., Toronto, ON, Canada) and subsequent daily injections as required. For all animals, water intake, urinary output, feed intake, and fecal output were monitored in metabolic cages on GD20, animals were sacrificed to collect maternal liver. Aliquots of whole blood were transferred to heparin- and thrombin-coated BD Vacutainer tubes (BD Canada, Mississauga, ON, Canada) and spun to collect plasma and serum, respectively. Samples were snap-frozen in liquid nitrogen and stored at −80°C until use.

Blood Chemistry. Determination of total cholesterol, high-density lipoprotein (HDL)/low-density lipoprotein (LDL) cholesterol, triglyceride, FFA, and albumin concentrations in serum was outsourced to the Banting and Best Diabetes Centre, University of Toronto. Total cholesterol, HDL/LDL cholesterol, triglyceride, and albumin concentrations in serum were outsourced to the Banting and Best Diabetes Centre, University of Toronto. Total cholesterol, HDL/LDL cholesterol, triglyceride, and albumin concentrations were analyzed by using an AutoAnalyzer and commercially available reagents (Roche Diagnostics, Laval, QC, Canada), whereas FFA was analyzed by using a commercially available enzyme-linked immunosorbent assay kit (Wako Chemicals USA, Inc.). Determination of alanine transaminase concentrations in plasma was outsourced to Vita-Tech Laboratories (Markham, ON,
Canada), where an AutoAnalyzer and commercially available reagents (Roche Diagnostics) were used. C-reactive protein (CRP) and AAG concentrations were determined in-house by using commercially available, rat-specific enzyme-linked immunosorbent assay kits (BD Biosciences, San Jose, CA and GenWay, San Diego, CA, respectively), according to the manufacturers’ instructions.

Protein Binding Assays. Glyburide (PubChem compound ID 3488) and saquinavir (PubChem compound ID 60787) protein binding in rat plasma was determined in vitro by ultrafiltration using the Centricon micropartition system (Millipore Canada, Ltd., Etobicoke, ON, Canada). Glyburide is an acidic drug that binds to albumin in plasma, and saquinavir is a basic drug that binds to AAG in plasma. Aliquots of [3H]glyburide (PerkinElmer Life and Analytical Sciences, Waltham, MA) or [3H]saquinavir (American Radiolabeled Chemicals, St. Louis, MO), dissolved in ethanol, were placed in microcentrifuge tubes and dried under nitrogen gas. Dried [3H]glyburide or [3H]saquinavir was then reconstituted with drug-free plasma from each of this study’s three groups (n = 5/group) to obtain a final glyburide or saquinavir concentration of 30 ng/ml or 0.5 μg/ml, respectively, in a final volume of 200 μl. Samples were then incubated at 36°C for 1 h with gentle agitation every 20 min. At 1 h, 150-μl aliquots were transferred to ultrafiltration tubes and centrifuged at 1000g for 30 min. After centrifugation, aliquots (25 μl) of filtered and unfiltered sample were supplemented with 4 ml of PicoFluor 40 (PerkinElmer Life and Analytical Sciences) and subjected to scintillation counting. Percentage unbound was determined by using the following formula: percentage unbound = (filtered count/unfiltered count) × 100. Preliminary experiments demonstrated constant glyburide and saquinavir protein binding across a wide range of concentrations. The final concentrations selected for plasma protein binding experiments reflect values within therapeutic ranges. To directly determine the capacity for plasma lipids to alter protein binding, total lipid content was removed from drug-free nontreated diabetic, insulin-treated diabetic, and vehicle control plasma samples (n = 5/group) using the butanol/disopropyl ether extraction method of Cham and Knowles (1976). This delipidation procedure removes 100% of plasma lipids without affecting the concentrations of other plasma constituents (Cham and Knowles, 1976). Delipidated samples were then subjected to the glyburide protein binding procedure as described. Reported values are the result of triplicate experiments and are reported as mean ± S.D.

Proteomics: Sample Preparation and Isobaric Mass Tagging. A proteomics approach (8Plex iTRAQ) was used to characterize the expression of hepatic proteins involved in lipid metabolism and/or drug (Supplemental Fig. 1). Our experimental design consisted of two experiments: a first experiment comparing four non-treated diabetic, insulin-treated diabetic, and vehicle control plasma samples (n = 5/group) using the butanol/disopropyl ether extraction method of Cham and Knowles (1976). This delipidation procedure removes 100% of plasma lipids without affecting the concentrations of other plasma constituents (Cham and Knowles, 1976). Delipidated samples were then subjected to the glyburide protein binding procedure as described. Reported values are the result of triplicate experiments and are reported as mean ± S.D.

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Proteomics: High-Performance Liquid Chromatography-Tandem Mass Spectrometry. Sample clean-up and fractionation was performed by strong cation exchange (SCX) chromatography on an Agilent Technologies (Forest Hill, Victoria, Australia) 1100 quaternary high-performance liquid chromatography (LC) pump with a PolySulfoethyl A column (2.1 mm × 200 mm, 5-μm particle size, 300-A pore) from PolyLC, Inc. (Columbia, MD). Two buffers (A and B) were used for SCX. Buffer A was 5 mM phosphate and 25% acetonitrile (pH 2.7), and buffer B was 5 mM phosphate, 350 mM potassium chloride and 25% acetonitrile (pH 2.7). Combined, iTRAQ-labeled samples were resuspended in 14 ml of buffer A. This solution was filtered, and 7 ml was then loaded onto the SCX column. After sample loading and washing with buffer A, the concentration of buffer B was increased from 10 to 45% in 70 min and then increased to 100% for 10 min at 300 μl/min. For each experiment, a total of 23 SCX fractions were collected and dried by SpeedVac before storage at −20°C to await mass spectrometry (MS). Reverse-phase nanoLC electrospray ionization tandem mass spectrometry (MS/MS) was conducted on a QStar Elite MS/MS system (Applied Biosystems, Foster City, CA). SCX fractions were resuspended in 60 μl of a loading/desalting solution consisting of 0.1% trifluoroacetic acid and 2% acetonitrile. Forty microliters of this solution was loaded on a reverse-phase peptide Cap Trap (Michrom Bioresources, Inc., Auburn, CA) desalted with 0.1% formic acid and 2% acetonitrile at 8 μl/min for 13 min. The trap was then switched online with a ProteoCol C18 trap cartridge (150 μm × 100 mm, 3-μm particle size, 300-A pore) from SGE Analytical Science (Ringwood, Victoria, Australia). Channel 1 contained desalting solution, channel 2A contained 0.1% formic acid, and channel 2B contained 0.1% formic acid and 90% acetonitrile. The concentration of solution in channel 2 was increased from 5 to 95% in 95 min in three linear gradient steps to elute peptides. After elution, the column was cleaned by increasing the concentration of channel 2B from 0 to 100% for 20 min followed by equilibration with 95% channel 1 and 5% channel 2B for 7 min before injection of the next SCX fraction.

Reverse-phase nanoLC eluent was subjected to positive ion nano-flow electrospray analysis in an information-dependent acquisition mode (Applied Biosystems). In this mode, a time-of-flight MS survey scan was acquired (m/z 370–1600, 0.5 s) with the three most intense multiply charged ions (counts >50) in the survey scan sequentially subjected to MS/MS analysis. Spectra were accumulated for 2 s in the range of m/z 100 to 1600 with a modified Q2 transition setting favoring low mass ions so that iTRAQ ion (113, 114, 115, 116, 117, 118, 119, and 121) intensities were enhanced.

Proteomics: Database Processing and Analysis. Experimental nanoLC electrospray ionization MS/MS data were submitted to ProteinPilot (Applied Biosystems, version 2.0) for database processing. The Paragon Algorithm was used in a thorough ID search effort with biological modifications selected in the ID focus box. Software correction factors, provided in the iTRAQ kit, were entered in the iTRAQ isotope correction factors table. The detected protein threshold (unused ProtScore) was set as larger than 1.3, which corresponds to an identification confidence of 95%. The unused ProtScore estimates the confidence associated with identifications and uses only peptide evidence that is not better explained by a higher-ranking protein. Significantly altered proteins (see Statistics), up-regulated or down-regulated more than 10%, were imported into Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Inc., http://www.ingenuity.com). IPA software was used to identify protein networks and generate lists of proteins associated with specific molecular and cellular functions. IPA software was also used to graphically illustrate the molecular relationships between proteins on our lists and proteins stored in the Ingenuity Knowledge Base (IKB). In those figures, molecules are represented as nodes, and the biological relationships between nodes are represented as connecting lines; lines are supported by at least one reference from IKB, which is based on the literature or canonical information stored in the IKB. IPA software was then used to compare all altered proteins in our experiments to tox lists in the IKB. Tox lists are lists of molecules that are known to be involved in particular aspects of toxicity, and our focus was on lists that addressed nuclear receptor activation.

Characterization of Hepatic Mdr1 Expression. In rodents, two genes encode the Mdr1 protein: Mdr1a (Abcb1a) and Mdr1b (Abcb1b). In this study, Mdr1a and Mdr1b mRNA levels and Mdr1 protein levels were determined in liver. For mRNA levels, total RNA was filtered, and 7 ml was then loaded onto the SCX column. After sample loading and washing with buffer A, the concentration of buffer B was increased from 10 to 45% in 70 min and then increased to 100% for 10 min at 300 μl/min. For each experiment, a total of 23 SCX fractions were collected and dried by SpeedVac before storage at −20°C to await mass spectrometry (MS).

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was extracted from tissue by using the TRIzol method (Invitrogen, Carlsbad, CA) and then reverse-transcribed to cDNA via the First Strand cDNA synthesis kit (Fermentas, Burlington, ON, Canada), according to the manufacturer's instructions. mRNA levels were determined by quantitative reverse transcriptase-polymerase chain reaction (PCR) using LightCycler technology with SYBR Green I fluorescence detection (Roche Diagnostics). PCR oligonucleotides were synthesized at The Hospital for Sick Children, DNA Synthesis Centre, Toronto, ON, Canada. Their sequences have been published previously (Petrovic et al., 2008). mRNA levels were normalized to 18S rRNA and expressed as a gene/18S ratio.

For Mdr1 protein levels, tissue samples were first homogenized in RIPA buffer (Cell Signaling Technologies, Pickering, ON, Canada), containing freshly added phenylmethylsulfonyl fluoride and 1× protease inhibitor cocktail (Sigma-Aldrich), using a motorized pestle. Homogenates were then incubated at 4°C for 2 h on a rocker and then centrifuged at 14,000 rpm for 20 min. For each sample, the supernatant was isolated and subjected to Bradford assay to determine total protein concentration. Samples containing 60 μg of protein in Laemmli sample buffer were heated at 36°C for 20 min and then separated via 10% SDS-polyacrylamide gel electrophoresis. Protein in Laemmli sample buffer were heated at 36°C for 20 min and then separated via 10% SDS-polyacrylamide gel electrophoresis. The effect of STZ-induced GDM, with and without insulin treatment, on various physiological and biochemical characteristics (Table 1). Blood glucose concentrations first exceeded 14 mM and was maintained for 12 to 13 days because sacrifice occurred on GD20. For the induction of GDM in STZ-injected rats, the blood glucose concentrations exceeded 14 mM and then remained above 25 mM for 24 h. For the induction of GDM in STZ-injected rats, the blood glucose concentrations exceeded 14 mM and then remained above 25 mM for 24 h. All of the STZ-injected rats developed mild hyperglycemia within 24 h and then developed GDM (>14 mM blood glucose concentration) by GD9. All animals in the GDM group were, therefore, diabetic for 12 to 13 days because sacrifice occurred on GD20. For the insulin-treated group, insulin treatment was initiated when blood glucose concentrations first exceeded 14 mM and was efficacious within hours. Blood glucose concentrations in the GDM group, relative to controls, were higher at every time point after GD9 (p < 0.001; Table 1). Blood glucose concentrations in the insulin-treated group fluctuated but were lower than those in the GDM group.

**Results**

**STZ-Injected Pregnant Rats Exhibited a Variety of Characteristics That Were Consistent with Unmanaged or Poorly Managed GDM.** Induction of GDM in STZ-injected rats was determined on the basis of their blood glucose concentrations as measured on GDs 7–9, 11, 13, 15, 18, and 20 (Supplemental Fig. 2A). All of the STZ-injected rats developed mild hyperglycemia within 24 h and then developed GDM (>14 mM blood glucose concentration) by GD9. All animals in the GDM group were, therefore, diabetic for 12 to 13 days because sacrifice occurred on GD20. For the insulin-treated group, insulin treatment was initiated when blood glucose concentrations first exceeded 14 mM and was efficacious within hours. Blood glucose concentrations in the GDM group, relative to controls, were higher at every time point after GD9 (p < 0.001; Table 1). Blood glucose concentrations in the insulin-treated group fluctuated but were lower than those in the GDM group.

**TABLE 1**

The effect of STZ-induced GDM, with and without insulin treatment, on various physiological and biochemical characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Vehicle</th>
<th>GDM</th>
<th>Insulin-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose and body weight</td>
<td>6.2 ± 0.8</td>
<td>6.6 ± 0.9</td>
<td>6.5 ± 1.8</td>
</tr>
<tr>
<td>Blood glucose (mM, GD6)</td>
<td>5.8 ± 0.7</td>
<td>24.7 ± 3**</td>
<td>8.4 ± 3.2***</td>
</tr>
<tr>
<td>Blood glucose (mM, GD11)</td>
<td>5.1 ± 0.5</td>
<td>2 ± 2**</td>
<td>5.5 ± 2.3**</td>
</tr>
<tr>
<td>Blood glucose (mM, GD20)</td>
<td>5.4 ± 1</td>
<td>2 ± 2**</td>
<td>5.7 ± 1.9**</td>
</tr>
<tr>
<td>Body weight (g, GD6)</td>
<td>241 ± 23</td>
<td>242 ± 28</td>
<td>246 ± 22</td>
</tr>
<tr>
<td>Body weight (% baseline, GD11)</td>
<td>114 ± 2</td>
<td>194 ± 3**</td>
<td>112 ± 2**</td>
</tr>
<tr>
<td>Body weight (% baseline, GD18)</td>
<td>138 ± 7</td>
<td>124 ± 5**</td>
<td>136 ± 8***</td>
</tr>
<tr>
<td>Body weight (% baseline, GD20)</td>
<td>150 ± 8</td>
<td>137 ± 7**</td>
<td>150 ± 8*</td>
</tr>
<tr>
<td>Blood chemistry (GD20)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mM)</td>
<td>1.9 ± 0.4</td>
<td>5 ± 1.4**</td>
<td>2.2 ± 0.8*</td>
</tr>
<tr>
<td>HDL cholesterol (mM)</td>
<td>0.8 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>1.2 ± 0.1**</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>3.65 ± 1.54</td>
<td>19.15 ± 9.08*</td>
<td>2.12 ± 0.74*</td>
</tr>
<tr>
<td>Free fatty acids (mEq/l)</td>
<td>0.9 ± 0.3</td>
<td>2.5 ± 1.3</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>AAG (µg/ml)</td>
<td>25.2 ± 4.4</td>
<td>39.5 ± 5.8*</td>
<td>33.8 ± 8.5</td>
</tr>
<tr>
<td>Albumin (µg/l)</td>
<td>35.7 ± 3.4</td>
<td>21.8 ± 3.3**</td>
<td>39 ± 0.1**</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>54.8 ± 6.6</td>
<td>104.8 ± 20.4**</td>
<td>74.8 ± 22.7</td>
</tr>
<tr>
<td>CRP (µg/ml)</td>
<td>318.2 ± 33.5</td>
<td>309.2 ± 45.3</td>
<td>296.2 ± 58.1</td>
</tr>
<tr>
<td>Metabolic cage (GD18)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water intake (ml/kg/day)</td>
<td>152 ± 5</td>
<td>800 ± 201**</td>
<td>189 ± 133**</td>
</tr>
<tr>
<td>Urinary output (ml/kg/day)</td>
<td>78 ± 23</td>
<td>671 ± 214**</td>
<td>148 ± 85*</td>
</tr>
<tr>
<td>Feed intake (g/kg/day)</td>
<td>81.1 ± 28.2</td>
<td>177.6 ± 61.9</td>
<td>77.4 ± 21.5</td>
</tr>
<tr>
<td>Fecal output (g/kg/day)</td>
<td>20 ± 7.5</td>
<td>95.6 ± 35.2**</td>
<td>20.9 ± 0.2*</td>
</tr>
<tr>
<td>Urinalysis (GD18)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total protein output (mg/kg/day)</td>
<td>11.3 ± 6.5</td>
<td>35.2 ± 43.4</td>
<td>8.2 ± 1.1</td>
</tr>
</tbody>
</table>

All results are presented as mean ± S.D. of five rats/group. *, Significantly different from vehicle controls. #, Significantly different from GDM. **p < 0.05; ***p < 0.01; ****p < 0.001. All results are presented as mean ± S.D.
different from values obtained in the vehicle group only on GD13.

As expected during gestation, all animals gained weight across the study duration; however, animals with GDM were lighter from GD8 until sacrifice ($p < 0.01–0.001$; Table 1 and Supplemental Fig. 2B). On GD20, the body weight of animals in the GDM group was $136 \pm 7\%$ of baseline values versus the $150 \pm 8\%$ increase observed in the vehicle and insulin-treated groups ($p < 0.01$).

Animals in the GDM group exhibited other physiological and biochemical characteristics consistent with unmanaged, or poorly managed, GDM (Table 1). Hyperlipidemia was evident in the GDM group as plasma cholesterol (total) and triglyceride concentrations were profoundly elevated. FFA concentrations were also elevated, but this finding was not statistically significant ($p = 0.06$). In addition, animals in the GDM group exhibited obvious polydipsia and polyuria on GD18. While in metabolic cages, relative to the vehicle and insulin-treated groups, animals in the GDM group exhibited a 5.2- and 4.2-fold increase in water consumption, respectively, and a 8.6- and 4.6-fold increase in urination, respectively. Animals in the GDM group also exhibited hyperphagia with an associated increase in fecal output. Relative to controls, animals in the GDM group consumed over twice as much food and produced nearly five times as many feces. Plasma from animals in the GDM group contained higher triglyceride concentrations were also elevated, but this finding was not statistically significant ($p = 0.06$). In addition, animals in the GDM group exhibited hyperlipidemia as plasma cholesterol (total) and triglyceride concentrations were profoundly elevated. FFA concentrations were also elevated, but this finding was not statistically significant ($p = 0.06$). In addition, animals in the GDM group exhibited hyperphagia with an associated increase in fecal output. Relative to controls, animals in the GDM group consumed over twice as much food and produced nearly five times as many feces. Plasma from animals in the GDM group contained higher levels of AAG and lower levels of albumin, compared with the vehicle group. Albumin and AAG concentrations in the insulin-treated group were slightly elevated, but this finding was not statistically significant. Compared with the vehicle and insulin-treated groups, alanine transaminase was found to be elevated in the GDM group, whereas CRP values were found to be similar ($p > 0.05$).

**Decreased Plasma Protein Binding of Acidic and Basic Drugs Was Observed in GDM.** The plasma protein binding of glyburide, an acidic drug that binds to albumin, and saquinavir, a basic drug that binds to AAG, was examined. Unbound glyburide was found to be $13.4 \pm 2.6\%$ in plasma from animals with GDM, which was higher than controls: $5.2 \pm 2.6\%$ in the vehicle group and $3.8 \pm 0.9\%$ in the insulin-treated group ($p < 0.0001$; Fig. 1A). For saquinavir, $12.2 \pm 1.1\%$ was unbound in plasma from animals with GDM, which was again higher than controls: $9.7 \pm 1.2\%$ in the vehicle group and $8.9 \pm 1.1\%$ in the insulin-treated group ($p < 0.01$; Fig. 1B). Although the unbound percentages in the insulin-treated group were lower than those in the vehicle group, the magnitude of these changes was quite modest. Linear regression revealed a positive relationship between the plasma protein binding of glyburide and saquinavir ($r^2 = 0.85; p < 0.0001$; Fig. 1C).

**Evidence for Hyperlipidemia as a Mechanism for Decreased Plasma Protein Binding in GDM.** To determine the contribution of plasma lipids to the altered protein binding observed in the GDM group, plasma samples were delipidated before analysis of plasma protein binding capacity. As illustrated in Fig. 1D, delipidation of plasma from the GDM group dramatically reduced the percentage of unbound glyburide from $13.4 \pm 2.6\%$ to $6.1 \pm 0.3\%$ ($p < 0.01$). Postdelipidation protein binding in the GDM group was not statistically different from postdelipidation protein binding in the vehicle group ($p > 0.05$). Delipidation had no effect on the protein binding of glyburide in the vehicle or insulin-treated groups ($p > 0.05$).

**Alterations in the Expression of Hepatic Membrane Proteins in GDM Were Largely Linked to Lipid Metabolism and Were Both Prohyperlipidemic and Antihyperlipidemic.** Proteomic analysis consisted of two iTRAQ experiments: experiment 1 compared the GDM and vehicle groups, and experiment 2 compared the insulin-treated and vehicle groups. Experiment 1 detected 625 hepatic proteins, 142 of which were significantly altered (Fig. 2A), and experiment 2 detected 607, 125 of which were significantly altered.
were normalized by insulin treatment, 22% (7) lacked com-
in lipid metabolism (Table 2). Of these proteins, 72% (23)
(32) were identified as having a molecular/cellular function
by IPA software. Of the 142 proteins altered in GDM, 22.5%
the top five molecular networks that were identified in GDM
bolic enzymes known to be present in rat liver.

Evidence for Nuclear Receptor Activation Contrib-
uting to Drug Transporter and Metabolic Enzyme
Up-Regulation. The networks depicted in Figs. 3 and 4
depict molecular networks for these proteins, illustrating the
connections between proteins quantified in our experiments
and a myriad of proteins implicated on the basis of IKB data.

Fig. 2. Volcano plots depicting the magnitude and statistical significance
of hepatic protein alterations in iTRAQ experiments. Negative log p
values for all 625 proteins identified in the GDM versus vehicle group
comparison (A) and all 607 proteins identified in the insulin-treated
versus vehicle group comparison (B) were plotted against log fold change.

Members of the ATP-binding cassette (ABC) and solute
carrier organic anion transporters, cytochrome P450 (CYP)
enzymes and UDP glucuronosyltransferases (UGTs) were
present in both data sets; however, owing probably to the
extensive sharing of peptide sequences, we positively identi-
fied only a modest fraction of drug transporters and meta-
bolic enzymes known to be present in rat liver.

Altered lipid metabolism appeared as a function in two of
the top five molecular networks that were identified in GDM
by IPA software. Of the 142 proteins altered in GDM, 22.5%
(32) were identified as having a molecular/cellular function
in lipid metabolism (Table 2). Of these proteins, 72% (23)
were normalized by insulin treatment, 22% (7) lacked com-
parison data in the insulin treatment group, and 6% (2) were
similarly altered.

A review of the literature concerning the proteins listed
in Table 2 indicated that alterations in the GDM group
were both prohyperlipidemic and antihyperlipidemic. For
the purposes of presenting and discussing results, alter-
ations were considered prohyperlipidemic if they would be
expected to increase the hepatic synthesis of lipids or
promote their retention in the blood compartment. Con-
versely, alterations were considered antihyperlipidemic if
they would be expected to decrease the hepatic synthesis of
lipids or promote their retention in nonblood compart-
ments (e.g., within hepatocytes). The up-regulation of
FDFT1 and CYP51A1 in GDM are examples of prohyper-
lipidemic alterations. Examples of antihyperlipidemic al-
terations in GDM include the down-regulation of ACACA,
ACAT1, and FASN and the up-regulation of ACADL. The
expression of lipid transporting proteins was also altered
in GDM. CD36 was down-regulated, whereas ABCB4
(Mdr2) and solute carrier organic anions 27A2 and 27A5
were up-regulated.

It is noteworthy that lipid metabolism did not appear as a
function in any of the major molecular networks that were
identified in the insulin-treated group. Despite this, alter-
ations in 14 proteins involved in lipid metabolism were
found in the insulin-treated group’s proteomics data set (Table 3).
As with GDM, alterations in the insulin-treated group were
both prohyperlipidemic and antihyperlipidemic. The down-
regulation of ABCD3, ABCG2, and CD36 in this group are
examples of prohyperlipidemic alterations. Examples of
antihyperlipidemic alterations after insulin treatment in-
clude the finding that emopamil binding protein and
SOAT2 were down-regulated and the finding that LDLR
was up-regulated.

Several proteins involved in drug transport and metabolism
were up-regulated in GDM, including: ABCB4, CYP4A11,
CYP4A14, CYP51A1, and UGT1A1. In the insulin-treated group,
ABCD3, ABCG2, and CYP2B6 were down-regulated, whereas
CYP4F8 and xanthine dehydrogenase were up-regulated.
OATP1A1 was down-regulated in both groups.

To investigate the relationships specifically between pro-
teins involved in lipid metabolism, all altered proteins asso-
ciated with this function in experiments 1 and 2 were isolated
and individually subjected to IPA analysis. Figures 3 and 4
depict molecular networks for these proteins, illustrating the
connections between proteins quantified in our experiments
and a myriad of proteins implicated on the basis of IKB data.
In Figs. 3 and 4, one feature that stands out is the presence
of network “hubs” that are comprised of nuclear receptors
(these hubs appear in blue).

Evidence for Nuclear Receptor Activation Contrib-
uting to Drug Transporter and Metabolic Enzyme
Up-Regulation. The networks depicted in Figs. 3 and 4
highlight the involvement of nuclear receptors with roles
in lipid metabolism and the regulation of drug transport-
ers and metabolic enzymes. These networks implicated
involvement of FXR, liver X receptor (LXR), nuclear factor
κB, peroxisome proliferator-activated receptors (PPARs),
and thyroid hormone receptor in GDM-mediated alter-
ations. On the other hand, network analysis implicated
hepatic nuclear factor 1 α and β (HNF1A/HNF1B), nuclear
factor-κB, and PPARγ in alterations that were observed in
the insulin-treated group. Given the apparent activity of these nuclear receptors, at least within the subset of our data concerned with lipid metabolism, we decided to compare all altered proteins observed in both iTRAQ experiments to nuclear receptor activation tox lists in the IKB (Table 4). At present, tox lists are available for the activation of FXR, LXR, PPARs, and PXR as well as the constitutive androstane receptor (CAR) and nuclear factor erythroid 2-related factor 2. With the exception of CAR, relative to animals in the insulin-treated group, animals in
the GDM group consistently exhibited a higher number of alterations to proteins associated with the activation of these nuclear receptors (22 unique proteins versus 10 unique proteins). As predicted, many of the specific proteins that overlapped with nuclear receptor activation tox lists were transporters, CYPs, or other proteins of relevance to drug transport and metabolism. It is important to note that the majority of proteins presented in Table 4 were identified in both iTRAQ experiments.

Table 4 also includes data from two additional lists: 1) CYPs with xenobiotic substrates and 2) xenobiotic metabolism. Including these additional lists revealed a handful of differentially expressed proteins that did not appear in lipid metabolism networks or the nuclear receptor activation tox lists. For the GDM group, the additional lists identified CYP2A1, CYP2B9, and CYP2D3 as xenobiotic metabolizing enzymes. All three proteins were up-regulated in GDM. For the insulin-treated group, the additional lists identified ALDH1L1, glutathione S-transferase A4 and Mu 1 (GSTA4 and GSTM1) as well as UGT1A4 as xenobiotic metabolizing enzymes. UGT1A4 was down-regulated, whereas ALDH1L1, GSTA4, and GSTM1 were up-regulated.

Because our evidence suggested activation of FXR and PXR via the up-regulation of their target genes (e.g., Mdr2), the FXR/PXR “target gene” Mdr1 was examined at the level of mRNA and protein. Mdr1 was absent from both iTRAQ data sets, and this supplementary analysis allowed us to further test our hypothesis that hyperlipidemia would have indirect effects on drug transporters. As Fig. 5 illustrates, Mdr1b/18S standard ratios were higher in GDM at 0.26 ± 0.15 compared with 0.01 ± 0.004 in the vehicle group and 0.03 ± 0.02 in the insulin-treated group (p < 0.01). Mdr1/β-actin standard ratios were also higher in GDM at 0.41 ± 0.1 compared with 0.23 ± 0.07 in the vehicle group and 0.22 ± 0.06 in the insulin-treated group (p < 0.01). All groups had comparable Mdr1α/18S standard ratios (p > 0.05).
Comparisons of proteins that were altered in iTRAQ experiments to tox lists in the Ingenuity Knowledge Base

Data sets were examined by IPA software for overlap with tox list proteins. Tox lists are lists of molecules that are known to be involved in particular aspects of toxicity, and our focus was on lists that addressed nuclear receptor activation and xenobiotic metabolism. Underlined entries were not common to the datasets of both experiments.

<table>
<thead>
<tr>
<th>Tox List ID (Total number of list members)</th>
<th>STZ</th>
<th>STZ + Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAR/RXR activation (28)</td>
<td>UGT1A1, ABCB4, APOA1, G6PC, SLC27A5</td>
<td>FASN</td>
</tr>
<tr>
<td>FXR/RXR activation (66)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>LXR/RXR activation (55)</td>
<td>APOA1</td>
<td>ACACA, CD36, FASN</td>
</tr>
<tr>
<td>Nrf2 activation (148)</td>
<td>CYP2A1, CYP4A11, CYP4A14</td>
<td>—</td>
</tr>
<tr>
<td>PPARα/RXR activation (118)</td>
<td>ACADL, APOA1, ALDH1A7, G6PC, HMGC52, UGT1A1</td>
<td>CD36, FASN</td>
</tr>
<tr>
<td>FXR/RXR activation (57)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cytochrome P450 panel (rat)–substrate is a xenobiotic (23)</td>
<td>CYP2A1, CYP2B3</td>
<td>—</td>
</tr>
<tr>
<td>Xenobiotic metabolism (89)</td>
<td>CYP2A1, CYP2B9, CYP2D3, CYP51A1, UGT1A1</td>
<td>—</td>
</tr>
</tbody>
</table>

Fig. 4. Lipid metabolism network in the insulin-treated group. IPA software was used to graphically illustrate the molecular relationships between altered proteins that were linked to lipid metabolism and proteins stored in the IKB. Molecules are represented as nodes, and the biological relationships between nodes are represented as connecting lines. Molecules are labeled with their Entrez gene symbols (see Tables 2 and 3). Up-regulated proteins are red, down-regulated proteins are green, nuclear receptors are blue, and proteins inserted by IPA software are white.

TABLE 4
Comparisons of proteins that were altered in iTRAQ experiments to tox lists in the Ingenuity Knowledge Base

Data sets were examined by IPA software for overlap with tox list proteins. Tox lists are lists of molecules that are known to be involved in particular aspects of toxicity, and our focus was on lists that addressed nuclear receptor activation and xenobiotic metabolism. Underlined entries were not common to the datasets of both experiments.
protein binding of glyburide should not have been completely normalized by delipidation alone. Moreover, delipidation did not affect glyburide protein binding in controls. Taken together, our results provide strong evidence in support of hyperlipidemia contributing directly to decreased plasma protein binding of drugs in GDM.

In our proteomics experiments, many alterations in GDM occurred in proteins that are linked to lipid metabolism. Although studies have associated the etiology of hyperlipidemia in STZ-induced diabetes with decreased insulin signaling and altered fat absorption, the contribution of hepatic proteins has remained largely unexplored.

Based on our findings in GDM, there seem to be a number of prohyperlipidemic and antihyperlipidemic responses in the liver, and some of these changes could greatly influence plasma lipids. One particularly interesting prohyperlipidemic response was the up-regulation of FDFT1 and CYP51A1 in GDM, both of which are proteins that play critical roles in the conversion of acetyl-CoA to cholesterol. In fact, FDFT1 is a potential therapeutic target for hyperlipidemia and an FDF1 inhibitor, lapuquistat/TAK-475, has been shown to be efficacious in preclinical and clinical studies (Nishimoto et al., 2003; Piper et al., 2006). Many of the prohyperlipidemic responses we observed are probably the result of excess acetyl-CoA being shunted to alternative routes of utilization. It is possible that, in GDM, enhanced hepatic lipid production is preferential to excess blood glucose.

With respect to interesting antihyperlipidemic hepatic responses in GDM, ACACA, ACAT1, and FASN were down-regulated, and ACADL was up-regulated. ACACA catalyzes the carboxylation of acetyl-CoA to produce malonyl-CoA, a substrate for the biosynthesis of fatty acids. ACAT1 catalyzes the esterification of cholesterol, which is subsequently incorporated into LDL and secreted. FASN’s main function is to catalyze the synthesis of acetyl-CoA and malonyl-CoA into long-chain saturated fatty acids. Decreased expression of ACACA, ACAT1, and FASN in GDM would, therefore, be expected to decrease the synthesis and secretion of lipids by the liver. ACADL catalyzes the initial step in mitochondrial fatty acid oxidation, whereby fatty acids are broken down to generate acetyl-CoA. This increased expression of ACADL in GDM would be expected to increase fatty acid utilization. Although interesting, it is important to note that these anti-hyperlipidemic hepatic responses, and those involving lipid transporting proteins, did not correct the hyperlipidemia present in our model of GDM. Thus, the regulation of proteins linked to lipid metabolism is pathologically disrupted in GDM. Studies to determine the relative contribution of the prohyperlipidemic and antihyperlipidemic alterations described here are warranted.

An indirect effect of dysregulation in lipid metabolism regulatory pathways, and one for which this study provides support, is an activation of nuclear receptor networks that have roles in the regulation of hepatic drug transporters and metabolic enzymes. Studies have demonstrated that several nuclear receptors have lipids as natural ligands (Handschin and Meyer, 2005). Activation of several of these nuclear receptors is consistent with our observation that many of the drug transporters and metabolizing enzymes that we identified were up-regulated in GDM. Activation of FXR, for example, is consistent with the up-regulation of ABCB4 (Mdr2) observed in GDM. FXR also regulates, in part, Mdr1 (Sted-
man et al., 2006), and up-regulation of this important drug transporter was observed in GDM. Another example is the activation of CAR and PXR, which is consistent with the up-regulation of UGT1A1 and CYP2A1 observed in GDM. These findings complement a body of literature demonstrating increased drug-metabolizing capacities in experimental animals and humans with diabetes (Barnett et al., 1999; Song et al., 1990). It is noteworthy that OATP1A1, which can be down-regulated in response to CAR, nuclear factor erythroid 2-related factor 2, or PPARs activation (Cheng et al., 2005; Maher et al., 2006), was found to be down-regulated in the GDM and insulin-treated groups.

The finding that several ABC transporters were up-regulated in GDM is contrary to our previous report in nonpregnant, female rats with acute STZ-induced diabetes, which demonstrated that the hepatic expression of Mdr1a, Abcc2, and Abcg2 mRNA was down-regulated (Anger et al., 2009). In that study, rats were diabetic for half as long as the rats in this study and had not yet developed overt hyperlipidemia, as measured on a lipemia index (unpublished data). Moreover, in our previous report we provided preliminary evidence that inflammation plays a role in the observed down-regulation, but inflammatory responses are often blunted during late pregnancy (Aguilar-Valles et al., 2007), which is supported by the fact that plasma CRP was not elevated in GDM. We speculate that the differences between this study and our previous study stem from an absence of hyperlipidemia and the presence of inflammation in the latter. It would be interesting to compare the results obtained in our GDM model with those of a model of pregnancy complicated by maternal type 1 diabetes, wherein the total duration of maternal diabetes could be extended even further.

Our proteomics findings in the insulin-treated group deserve further comment. First, insulin treatment did not normalize the expression of all proteins that were altered in GDM. Some of the changes in the insulin-treated group could be caused by our insulin treatment protocol. Daily injections of long-acting insulin lead to oscillating glucose concentrations with the potential for brief periods of hyperglycemia preinjection and hypoglycemia postinjection. It is possible that this was sufficient to stimulate some of the observed changes, both those shared with the GDM group and those unique to the insulin-treated group. Although hyperlipidemia is unlikely to have been transiently induced by these brief periods of abnormal blood glucose, some of the nuclear receptors discussed here are also capable of sensing glucose. LXR is an example (Mitro et al., 2007).

Second, insulin treatment itself probably affected the expression of hepatic proteins because there were proteins that were altered in the insulin-treated group that were not altered in GDM. Prohyperlipidemic alterations in the insulin-treated group that did not occur in GDM included the down-regulation of Abcd3. Abcd3 is involved in the transport of lipids, and down-regulation of this protein would be expected to prevent the movement of plasma lipids to hepatic routes of metabolism or elimination. Insulin-mediated antihyperlipidemic alterations that did not occur in GDM included the down-regulation of SOAT2 and the up-regulation of LDLR. SOAT2 produces intracellular cholesterol esters from long-chain fatty acyl-CoA and cholesterol. As noted with ACAT1, esterified cholesterol in the liver is subsequently incorporated into LDL and secreted. LDLR expression and activity is a major determinant of plasma cholesterol content, by binding to LDL and transporting it across membranes, and its up-regulation in the insulin treatment group would be expected to play an antihyperlipidemic role. An explanation for these alterations not being observed in the GDM group could be that some of these proteins respond to insulin levels and are, consequently, sensitive to the exogenous administration of insulin. LDLR is such a protein (Wade et al., 1989). Returning to plasma protein binding in our model of GDM, albumin production is also stimulated by insulin, which could explain the slightly elevated albumin concentrations and slightly increased protein binding observed in insulin-treated rats (Lloyd et al., 1987).

In conclusion, our findings demonstrate that hyperlipidemia in GDM contributes directly to decreased protein binding of drugs and indirectly to drug transporter and metabolic enzyme up-regulation. Our findings advance our understanding of the impact that GDM-induced hyperlipidemia can have on these specific drug disposition mechanisms. As mentioned, protein binding strongly influences a drug’s distribution and/or clearance. With distribution, placental transfer of drugs and the uptake of drugs by the liver and kidneys could significantly increase. Elevated hepatic drug transporters and metabolizing enzymes in GDM would be expected to compound changes in clearance that relate to the elevated uptake of drugs by the liver and kidneys. If confirmed in clinical populations, the effects reported in this study would need to be considered when atypical therapeutic outcomes are observed in pregnancies that are complicated by GDM.

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References


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