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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FXR; free fatty acid, FFA; gestational day, GD; gestational diabetes, GDM; hepatic nuclear factor 1, HNF1; high-density lipoprotein, HDL; high performance liquid chromatography, HPLC; Ingenuity Knowledge Base, IKB; low-density lipoprotein, LDL; mass spectrometry, MS; multidrug resistance 1/2, Mdr1/2; nuclear factor erythroid 2-related factor 2, Nrf2; nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB; organic anion transport protein, OATP; peroxisome proliferator-activated receptor, PPAR; pregnane x receptor, PXR; quantitative reverse transcriptase-polymerase chain reaction, qRT-PCR; solute carrier organic anion transporter, SLC; streptozotocin, STZ; strong cation exchange, SCX; thyroid hormone receptor, TR; UDP glucuronosyltransferase, UGT. Full Entrez gene names are presented alongside their symbols and UniProt identifiers in Tables 2 and 3.

Recommended assignment: Endocrine and diabetes
Abstract

It is currently unknown whether gestational diabetes (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 if excess lipids compete with drugs for plasma protein binding. Since lipids activate nuclear receptors that regulate drug transporters and metabolic enzymes, we used proteome analysis to determine if 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 using iTRAQ 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 dataset, with pro- and anti-hyperlipidemic changes observed, and formed networks that implicated several nuclear receptors. Upregulation of drug transporters and metabolic enzymes was observed (e.g., Mdr1/2, CYP2A1, CYP2B9 and CYP2D3). In this study, GDM-induced hyperlipidemia decreased protein binding and was associated with drug transporter and metabolic enzyme upregulation in the liver. Both of these findings could change drug disposition in affected pregnancies, compounding changes associated with pregnancy itself.
Introduction

Gestational diabetes (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. While it has been established that pregnancy itself decreases the plasma protein binding of drugs (Perucca et al., 1981; Notarianni, 1990; Tsen et al., 1999), there have been few attempts to determine if maternal diseases like 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 and so altered protein binding in pregnancy can significantly impact the materno-fetal transfer of drugs (Nanovskaya et al., 2006). With clearance, the removal of drugs by the liver and kidneys, processes that depend upon drug transporters and metabolic enzymes, often varies with plasma protein binding since uptake by these organs is also limited to unbound drugs. Atypical clearance has fetal implications in the form of changing the absolute amount of drug that is available for placental transfer and maternal implications in the form of diminished therapeutic effect (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 alpha 1-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; Reviewed in 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 utilizing 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 first removed (Chauvelot-Moachon et al., 1988). In pregnancy, diabetes exacerbates the natural rise in plasma lipids that occurs across pregnancy (Couch et al., 1998; Reviewed in 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 non-pregnant rats have demonstrated correlations between plasma lipids and the unbound percentage of both acidic and basic drugs following 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 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 pregnane 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.
**Methods**

**Animals.** Pregnant Sprague-Dawley rats (207-260 g) were purchased from Charles River Laboratories, Inc. (Senneville, QC) and housed individually in a temperature controlled facility on a 12:12 hr light-dark cycle. Rats were given free access to water and standard chow (Harlan Teklad 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 (referred to as the, “GDM group”), vehicle controls (referred to as the, “vehicle group”) and insulin-treated diabetics (referred to as 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 streptozotocin (STZ, PubChem Compound ID 5300, Sigma-Aldrich, Oakville, ON) 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 prior to respective injections (GD6) and then daily until sacrifice. For animals in the GDM and vehicle groups, blood glucose was measured prior to respective injections (GD6) and on gestational days 7, 8, 9, 11, 13, 15, 18 and 20. For animals in the insulin-treated group, blood glucose was measured prior to STZ injection (GD6) and then daily until sacrifice in order to determine daily insulin dosage requirements. Blood glucose was measured at noon, in a non-fasted state, using a commercially available glucometer (Freestyle
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 employed according to the manufacturer’s instructions (Autokit Glucose, Wako Chemicals USA, Inc., Richmond, VA). Mild hyperglycemia was defined as 10-14 mM and diabetes was defined as > 14 mM. For diabetic rats receiving insulin treatment, treatment was initiated once diabetes was confirmed and consisted of an initial subcutaneous injection of 8-10 U/kg Humulin-L (human biosynthetic intermediate-acting insulin, Lilly, Toronto, ON) and subsequent daily injections as required. For all animals, water intake, urinary output, feed intake and fecal output were monitored in metabolic cages on GD18. Following measurement of blood glucose and body weight on GD20, animals were sacrificed to collect maternal blood (cardiac puncture) and liver. Aliquots of whole blood were transferred to heparin- and thrombin-coated BD Vacutainer tubes (BD Canada, Mississauga, ON) 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 (Faculty of Medicine, University of Toronto). Total cholesterol, HDL/LDL cholesterol, triglyceride and albumin concentrations were analyzed using an autoanalyzer and commercially available reagents (Roche Diagnostics, Laval, QC) while FFA was analyzed using a commercially available ELISA kit (Wako Chemicals USA, Inc.). Determination of alanine transaminase (ALT) concentrations in plasma was outsourced to
Vita-Tech Laboratories (Markham, ON), where an autoanalyzer and commercially available reagents (Roche Diagnostics) were employed. C-reactive protein (CRP) and AAG concentrations were determined in-house using commercially available, rat-specific ELISA kits (BD Biosciences, San Diego, CA and GenWay, San Diego, CA, respectively), according to manufacturer’s 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 Centrifree micropartition system (Millipore Canada, Ltd., Etobicoke, ON). Glyburide is an acidic drug that binds to albumin in plasma while saquinavir is a basic drug that binds to AAG in plasma. Aliquots of [3H]glyburide (Perkin Elmer, Boston, MA) or [3H]saquinavir (American Radiolabeled Chemicals, Inc., 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 hr with gentle agitation every 20 min. At 1 hr, 150 μl aliquots were transferred to ultrafiltration tubes and centrifuged at 1000 g for 30 min. Following centrifugation, aliquots (25 μl) of filtered and unfiltered sample were supplemented with 4 ml of Pico-Fluor 40 (Perkin Elmer) and subjected to scintillation counting. Percent unbound was determined using the following formula: Percent 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. In order to directly determine the capacity for plasma lipids to alter protein binding, total lipid content was removed from drug-free non-treated diabetic, insulin-treated diabetic and vehicle control plasma samples (n = 5/group) using the butanol:diisopropyl ether extraction method of Cham and Knowles (Cham and Knowles, 1976). This delipidation procedure removes 100% of plasma lipids without impacting the concentrations of other plasma constituents (Cham and Knowles, 1976). Delipidated samples were then subjected to the glyburide protein binding procedure described in this methods section. Reported values are the result of triplicate experiments and are reported as mean ± SD.

**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 samples to three vehicle control samples and a second experiment comparing four insulin-treated diabetic samples to three vehicle control samples. In order to eliminate the contribution of blood proteins in these experiments, a proteomics-specific cohort of rats was randomly assigned to the treatments described above (n = 3-4/group, see Animal treatments/monitoring and tissue collection) but subjected, on GD20, to hepatic saline perfusion prior to liver collection (Mehta et al., 2009). Protein homogenates, obtained from perfused livers, underwent a membrane protein enrichment procedure as previously described (Molloy, 2008). For isobaric mass tagging, an 8Plex iTRAQ kit from Applied Biosystems
(Scoresby, Victoria, Australia) was used according to the manufacturer’s instructions. iTRAQ-labeled samples were combined (one combination per experiment) and then dried by SpeedyVac prior to storage at -20°C to await clean-up and fractionation.

**Proteomics: HPLC-MS/MS.** Sample clean-up and fractionation was performed by strong cation exchange (SCX) chromatography on an Agilent Technologies (Forest Hill, Victoria, Australia) 1100 quaternary HPLC pump with a PolySulfoethyl A column (2.1 mm × 200 mm, 5 μm particle size, 300 Å pore) from PolyLC, Inc. (Columbia, MA). Two buffers were used for SCX: A and B. 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 were 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 SpeedyVac prior to storage at -20°C to await mass spectrometry (MS).

Reverse phase nanoLC electrospray ionization (ESI) tandem mass spectrometry (MS/MS) was conducted on a QStar Elite MS/MS system (Applied Biosystems). SCX fractions were resuspended in 60 μl of a loading/desalting solution consisting of 0.1% trifluoroacetic acid and 2% acetonitrile. Forty μl of this solution were 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 Å 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 prior to injection of the next SCX fraction.

Reverse phase nanoLC eluent was subjected to positive ion nanoflow electrospray analysis in an information dependant 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 100-1600 with a modified Q2 transition setting favouring 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 ESI MS/MS data were submitted to ProteinPilot (Applied Biosystems, Version 2.0) for database processing. The Paragon Algorithm was used in 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), upregulated or downregulated greater 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 to 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 these figures, molecules are represented as nodes and the biological relationships between nodes are represented as connecting lines; lines are supported by at least 1 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 as well as Mdr1 protein levels were determined in liver. For mRNA levels, total RNA was extracted from tissue using the TRIZOL method (Invitrogen, Carlsbad, CA) and then reverse transcribed to cDNA via the First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON), according to the manufacturer’s instructions. mRNA levels were determined by quantitative reverse transcriptase-polymerase chain reaction (qRT-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) and their sequences have been previously published (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, containing freshly added PMSF and 1X protease inhibitor cocktail (Sigma-Aldrich), using a motorized pestle. Homogenates were then incubated at 4 °C for 2 hrs on a rocker and then centrifuged at 14,000 rpm for 20 min. For each sample, the supernatant was isolated and subjected to a 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-PAGE and transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories (Canada) Ltd.). Membranes were blocked in 5% fat-free milk powder at 4 °C overnight on a rocker and incubated with a mixture of monoclonal anti-Mdr1 (C219, 1:500, 1 mg/ml, Abcam Inc., Cambridge, MA) and anti-β-actin (AC-15, 1:20,000, 2 mg/ml, Sigma-Aldrich) antibodies at room temperature for 3 hrs. After a series of washes, membranes were incubated with a peroxidase-labeled secondary antibody for 1 hr at room temperature (1:3,000, goat anti-mouse from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Immunoreactive Mdr1 and β-actin proteins were simultaneously detected in membranes using ECL Plus (Amersham Biosciences, Baie d’Urfé, QC) and scanned using an Alpha Innotech FluorChem imaging system (San Diego, CA). Using AlphaEaseFC (Version 6.0) software, also from Alpha Innotech, the optical density (OD) of each band was determined. Mdr1 ODs were normalized to β-actin.
Statistics. Data were analyzed using Prism 4 for Macintosh (GraphPad Software, Inc., San Diego, CA). For comparisons of blood glucose and body weight, two-way ANOVAs followed by Bonferroni post-tests were performed. These same tests were employed to examine any potential differences between this study’s two cohorts, with respect to blood glucose and body weight. Protein binding of drugs (% unbound), plasma concentrations (e.g., individual lipids, albumin, etc.), hepatic Mdr1 expression data and metabolic cage variables were analyzed using one-way ANOVAs followed by Bonferroni post-tests. Linear regression was used to determine the relationship between the protein binding of glyburide and saquinavir. Paired T-tests were used to statistically evaluate the effects of plasma delipidation on the protein binding of glyburide. For proteomics data, ProteinPilot™ was used to generate control-standardized “fold changes” for each sample’s identified proteins. By standardizing to controls, the mean of control values was made to represent 1 ± SD while non-control values were made, consequently, to distribute around this value. Statistically significant deviations from control values were identified using T-tests. Levels of significance for all statistical analyses were set at or below $\alpha = 0.05$, indicated as follows: */# $p < 0.05$, **/## $p < 0.01$ and ***/### $p < 0.001$. All results are presented as mean ± SD.
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 GD7-9, 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) by GD9. All animals in the GDM group were, therefore, diabetic for 12 to 13 days since 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 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; Supplemental fig. 2B). On GD20, the body weight of animals in the GDM group was 136 ± 7% of baseline values versus the 150 ± 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). Also, 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 much feces. Plasma from animals in the GDM group contained higher levels of AAG and lower levels of albumin, as compared to the vehicle group. Albumin and AAG concentrations in the insulin-treated group were slightly elevated but this finding was not statistically significant. As compared to the vehicle and insulin-treated groups, ALT was found to be elevated in the GDM group while 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). While the unbound percentages in the insulin-treated group were lower than those in the vehicle group, the magnitude of these
changes were 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 prior to analysis of plasma protein binding capacity. As illustrated in figure 1D, delipidation of plasma from the GDM group dramatically reduced the percentage of unbound glyburide from $13.4 \pm 2.6\%$ to $6.1 \pm 0.3\% (p < 0.01)$. Post-delipidation protein binding in the GDM group was not statistically different from post-delipidation 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 pro- and anti-hyperlipidemic.** 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 (Fig. 2B). Comparison of the two iTRAQ datasets revealed a total of 476 proteins that were common: meaning that 149 proteins were detected only in the GDM versus vehicle comparison and 131 proteins were detected only in the insulin-treated versus vehicle comparison. The raw data associated with the proteomics component of this study may be
Members of the ATP-binding cassette (ABC) and solute carrier organic anion (SLC) transporters, cytochrome P450 (CYP) enzymes and UDP glucuronosyltransferases (UGTs) were present in both datasets; however, owing probably to the extensive sharing of peptide sequences, we positively identified only a modest fraction of drug transporters and metabolic 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 comparison 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 pro- and anti-hyperlipidemic. For the purposes of presenting and discussing results, alterations were considered pro-hyperlipidemic if they would be expected to increase the hepatic synthesis of lipids or promote their retention in the blood compartment. Conversely, alterations were considered anti-hyperlipidemic if they would be expected to decrease the hepatic synthesis of lipids or promote their retention in non-blood compartments (e.g., within hepatocytes). The upregulation of FDFT1 and CYP51A1 in GDM are examples of pro-hyperlipidemic alterations. Examples of anti-hyperlipidemic alterations in GDM include the
downregulation of ACACA, ACAT1 and FASN and the upregulation of ACADL. The expression of lipid transporting proteins was also altered in GDM. CD36 was downregulated while ABCB4 (Mdr2) and SLCs 27A2 and 27A5 were upregulated.

Interestingly, 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, alterations in 14 proteins involved in lipid metabolism were found in the insulin-treated group’s proteomics dataset (Table 3). As with GDM, alterations in the insulin-treated group were both pro- and anti-hyperlipidemic. The downregulation of ABCD3, ABCG2 and CD36 in this group are examples of pro-hyperlipidemic alterations. Examples of anti-hyperlipidemic alterations following insulin treatment include the finding that EBP and SOAT2 were downregulated and the finding that LDLR was upregulated.

Several proteins involved in drug transport and metabolism were upregulated in GDM, including: ABCB4, CYP4A11, 4A14 and 51A1 as well as UGT1A1. In the insulin-treated group, ABCD3, ABCG2 and CYP2B6 were downregulated while CYP4F8 and XDH were upregulated. Organic anion transport protein (OATP) 1A1 was downregulated in both groups.

To investigate the relationships specifically between proteins involved in lipid metabolism, all altered proteins associated with this function in experiment 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 as well as a myriad of proteins implicated on the basis of IKB data. In both figures 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 contributing to drug transporter and metabolic enzyme upregulation. The networks depicted in figures 3 and 4 highlight the involvement of nuclear receptors with roles in lipid metabolism as well as in the regulation of drug transporters and metabolic enzymes. These networks implicated involvement of FXR, liver x receptor (LXR), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), peroxisome proliferator-activated receptors (PPARs) and thyroid hormone receptor (TR) in GDM-mediated alterations. On the other hand, network analysis implicated hepatic nuclear factor 1 alpha and beta (HNF1A/HNF1B), NF-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 (Nrf2). 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 CYPs 2A1, 2B9 and 2D3 as xenobiotic metabolizing enzymes. All three proteins were upregulated in GDM. For the insulin-treated group, the additional lists identified ALDH1L1, GSTA4, GSTM1 and UGT1A4 as xenobiotic metabolizing enzymes. UGT1A4 was downregulated while ALDH1L1, GSTA4 and GSTM1 were upregulated.

Due to our evidence suggesting activation of FXR and PXR via the upregulation 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 datasets and this supplementary analysis allowed us to further test our hypothesis that hyperlipidemia would have indirect effects on drug transporters. As figure 5 illustrates, Mdr1b/18S standard ratios were higher in GDM at 0.26 ± 0.15 as compared to 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 as compared to 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 Mdr1a/18S standard ratios (p > 0.05).
Discussion

In this study, we employed the STZ rat model of GDM to determine if plasma lipids compete with drugs for protein binding in this disease. We also used this model to examine whether GDM is associated with alterations in hepatic drug transporters and metabolic enzymes.

With respect to plasma protein binding, we first determined the concentrations of two important plasma proteins: albumin and AAG. In humans, glyburide is 98% bound to albumin (Olsen et al., 1995) and was 95% protein bound in this study’s vehicle group. In humans, saquinavir is 98% bound to AAG (Holladay et al., 2001) and was 90% protein bound in this study’s vehicle group. In GDM, albumin concentrations were decreased and AAG levels were increased. This is consistent with clinical studies involving patients with poorly managed diabetes (Mattock et al., 2001).

If plasma protein concentrations were the sole determinant of protein binding then one would expect, based on albumin and AAG levels, decreased binding of glyburide and increased binding of saquinavir in GDM. However, in this study, the protein binding of both glyburide and saquinavir was lower in GDM than in the control groups. Moreover, as illustrated in figure 1C, the extent of one drug’s protein binding was found to be highly predictive of the other’s. This is indicative of a contribution from a factor other than absolute plasma protein content.

To test whether plasma lipids were, as hypothesized, competing with drugs for binding, we repeated our glyburide protein binding experiments with delipidated plasma samples. If plasma protein concentrations were the sole determinant of protein binding then one would also expect that delipidation would have no effect on protein binding;
however, this was not the case in this study. Delipidation completely normalized the protein binding of glyburide in GDM yet did not affect protein binding in either the vehicle or insulin-treated groups. It has previously been reported that the binding capacity of human albumin for glyburide was halved by protein glycosylation, a process which can occur in GDM (Koyama et al., 1997). If glycosylation was preventing glyburide protein binding in our study, however, the plasma 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. While 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 appear to be a number of pro- and anti-hyperlipidemic responses in the liver and some of these changes could greatly influence plasma lipids. One particularly interesting pro-hyperlipidemic response was the upregulation 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 FDFT1 inhibitor, lapaquistat/TAK-475, has been shown efficacious in preclinical and clinical studies (Nishimoto et al., 2003; Piper et al., 2006). Many of the pro-hyperlipidemic responses we observed are likely the result of
excess acetyl-CoA being shuttled 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 anti-hyperlipidemic hepatic responses in GDM, ACACA, ACAT1 and FASN were downregulated and ACADL was upregulated. 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. While interesting, it is important to note that these anti-hyperlipidemic hepatic responses, as well as 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 pro- and anti-hyperlipidemic 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 which 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 upregulated in GDM. Activation of FXR, for example, is consistent with the upregulation of ABCB4 (Mdr2) observed in GDM. FXR also regulates, in part, Mdr1 (Stedman et al., 2006) and upregulation of this important drug transporter was observed in GDM. Another example is the activation of CAR and PXR, which is consistent with the upregulation 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., 1990; Song et al., 1990). Interestingly, OATP1A1, which can be downregulated in response to CAR, Nrf2 or PPARα activation (Cheng et al., 2005; Maher et al., 2006), was found to be downregulated in the GDM and insulin-treated groups.

The finding that several ABC transporters were upregulated in GDM is contrary to our previous report in non-pregnant, female rats with acute STZ-induced diabetes, which demonstrated that the hepatic expression of Mdr1a, ABCC2 and ABCG2 mRNA was downregulated (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 downregulation 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 due to our insulin treatment protocol. Daily injections of long-acting insulin lead to oscillating blood glucose concentrations with the potential for brief periods of hyperglycemia pre-injection and hypoglycemia post-injection. 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. While 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 is likely to have affected the expression of hepatic proteins as there were proteins that were altered in the insulin-treated group that were not altered in GDM. Pro-hyperlipidemic alterations in the insulin-treated group that did not occur in GDM included the downregulation of ABCD3. ABCD3 is involved in the transport of lipids and downregulation of this protein would be expected to prevent the movement of plasma lipids to hepatic routes of metabolism or elimination. Insulin-mediated anti-hyperlipidemic alterations that did not occur in GDM included the downregulation of SOAT2 and the upregulation 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 upregulation in the insulin-treatment group would be expected to play an anti-hyperlipidemic 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 and this 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 upregulation. 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 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.
Acknowledgements

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References


Footnotes

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Legends for figures

Figure 1. Plasma protein binding characteristics. The plasma protein binding of glyburide/GLIB (A) and saquinavir/SQV (B) was determined in the GDM and control groups and expressed as percent unbound. C, Linear regression was used to determine the relationship between the binding of glyburide and saquinavir. D, Plasma samples were delipidated and then subjected to the glyburide protein binding procedure. All results are presented as mean ± SD of 5 rats/group; */#/p < 0.05, **/#/#p < 0.01 and ***/#/#/#p < 0.001. *Significantly different from vehicle controls, #Significantly different from GDM.

Figure 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 were plotted against log fold change. Blue horizontal lines correspond with $\alpha = 0.05$, meaning all proteins found above these lines are statistically different from controls.

Figure 3. Lipid metabolism network in the GDM 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. Upregulated proteins are red, downregulated proteins are green, nuclear receptors are blue and proteins inserted by IPA software are white.
Figure 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. Upregulated proteins are red, downregulated proteins are green, nuclear receptors are blue and proteins inserted by IPA software are white.

Figure 5. Hepatic Mdr1 expression. Hepatic Mdr1 was examined at the level of mRNA via qRT-PCR and at the level of protein via immunoblotting in the GDM and control groups. For mRNA, both genes encoding rodent Mdr1 were examined: Mdr1a and Mdr1b. All results are presented as mean ± SD of 5 rats/group; */#p < 0.05 and **/#p < 0.01. *Significantly different from vehicle controls, #Significantly different from GDM.
Table 1. The effect of STZ-induced GDM, with and without insulin treatment, on various physiological and biochemical characteristics. All results are presented as mean ± SD of 5 rats/group; */# \( p < 0.05 \), **/## \( p < 0.01 \) and ###/##\#\# \( p < 0.001 \). *Significantly different from vehicle controls, #Significantly different from GDM.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Vehicle</th>
<th>GDM</th>
<th>Insulin-treated</th>
</tr>
</thead>
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<tr>
<td><strong>Blood glucose and body weight:</strong></td>
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<td></td>
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</tr>
<tr>
<td>Blood glucose (mM, GD6)</td>
<td>6.2 ± 0.8</td>
<td>6.6 ± 0.9</td>
<td>6.5 ± 1.8</td>
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<td>Blood glucose (mM, GD11)</td>
<td>5.8 ± 0.7</td>
<td>24.7 ± 3.***</td>
<td>8.4 ± 3.2####</td>
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<td>Blood glucose (mM, GD18)</td>
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<td>26 ± 2***</td>
<td>5.5 ± 2.3####</td>
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<td>Blood glucose (mM, GD20)</td>
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<td>26 ± 2.***</td>
<td>5.7 ± 1.9####</td>
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<td>Body weight (g, GD6)</td>
<td>241 ± 23</td>
<td>242 ± 28</td>
<td>246 ± 22</td>
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<tr>
<td>Body weight (% baseline, GD11)</td>
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<td>104 ± 2***</td>
<td>112 ± 2####</td>
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<td>Body weight (% baseline, GD18)</td>
<td>138 ± 7</td>
<td>124 ± 5***</td>
<td>136 ± 8####</td>
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<td>Body weight (% baseline, GD20)</td>
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<td>150 ± 8####</td>
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<td><strong>Blood chemistry (GD20):</strong></td>
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<td>Total cholesterol (mM)</td>
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<td>5 ± 1.4**</td>
<td>2.2 ± 0.6##</td>
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<td>HDL cholesterol (mM)</td>
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<td>Triglycerides (mM)</td>
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<td>AAG (µg/ml)</td>
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<td>39.5 ± 5.8*</td>
<td>33.8 ± 8.5</td>
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<td>Albumin (g/L)</td>
<td>35.7 ± 3.4</td>
<td>21.8 ± 5.3**</td>
<td>39 ± 0.1##</td>
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<td>ALT (I.U./L)</td>
<td>54.8 ± 6.6</td>
<td>104.8 ± 20.4**</td>
<td>74.8 ± 22.7</td>
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<tr>
<td>CRP (µg/ml)</td>
<td>318.2 ± 33.5</td>
<td>305.2 ± 45.3</td>
<td>296.2 ± 58.1</td>
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</tbody>
</table>

**Metabolic cage (GD18):**

<p>| Water intake (ml/kg/day)              | 152 ± 5  | 800 ± 201** | 189 ± 133#### |
| Urinary output (ml/kg/day)            | 78 ± 23  | 671 ± 214** | 148 ± 85##       |
| Feed intake (g/kg/day)                | 81.1 ± 28.2 | 177.6 ± 61.9 | 77.4 ± 21.5     |</p>
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<td><strong>Fecal output (g/kg/day)</strong></td>
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<td>95.6 ± 33.2**</td>
<td>20.9 ± 0.2*</td>
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<td>Total protein output (mg/kg/day)</td>
<td>11.3 ± 6.5</td>
<td>35.2 ± 43.4</td>
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Table 2. IPA-identified proteins related to lipid metabolism in STZ-induced GDM. Control-standardized “fold changes” were generated for proteins linked to lipid metabolism. Differences of less than 10% were disregarded and statistically significant deviations from control values were identified using T-tests. \(*p < 0.05\), \(**p < 0.01\) and \(***p < 0.001\). NC: No change. Comparison values in the insulin-treated group were designated as NC when they were either not statistically different from those of controls or differed from controls by less than 10%.

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<th>UniProt</th>
<th>Symbol</th>
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<th>STZ Fold change</th>
<th>STZ + Insulin Coverage (%)</th>
<th>STZ + Insulin Fold change</th>
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<td>APOA1</td>
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<td>ATP5B</td>
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<td>P21571</td>
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<td>C3</td>
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<td>1.29*</td>
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<td>P61589</td>
<td>RHOA</td>
<td>Ras homolog gene family, member A</td>
<td>39.9</td>
<td>1.13*</td>
<td>43</td>
<td>NC</td>
</tr>
<tr>
<td>P17475</td>
<td>SERPINA1</td>
<td>Serpin peptidase inhibitor, clade A, member 1</td>
<td>46.7</td>
<td>1.16*</td>
<td>25.1</td>
<td>NC</td>
</tr>
<tr>
<td>P97524</td>
<td>SLC27A2</td>
<td>Solute carrier family 27 (fatty acid transporter), member 2</td>
<td>70.3</td>
<td>1.21***</td>
<td>67.6</td>
<td>NC</td>
</tr>
<tr>
<td>Q9ES38</td>
<td>SLC27A5</td>
<td>Solute carrier family 27 (fatty acid transporter), member 5</td>
<td>77.7</td>
<td>1.37**</td>
<td>52.2</td>
<td>NC</td>
</tr>
<tr>
<td>P24008</td>
<td>SRD5A1</td>
<td>Steroid-5-α-reductase, α polypeptide 1</td>
<td>35.5</td>
<td>-1.23***</td>
<td>57.9</td>
<td>NC</td>
</tr>
<tr>
<td>P61943</td>
<td>ST3GAL6</td>
<td>ST3 β-galactoside-α-2,3-sialyltransferase 6</td>
<td>19.9</td>
<td>-1.3*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Q64550</td>
<td>UGT1A1</td>
<td>UDP glucuronosyltransferase 1 family, polypeptide A1</td>
<td>63.2</td>
<td>1.3*</td>
<td>57.2</td>
<td>NC</td>
</tr>
</tbody>
</table>
Table 3. IPA-identified proteins related to lipid metabolism in insulin-treated STZ-induced GDM. Control-standardized “fold changes” were generated for proteins linked to lipid metabolism. Differences of less than 10% were disregarded and statistically significant deviations from control values were identified using T-tests. *p < 0.05, **p < 0.01 and ***p < 0.001. NC: No change. Comparison values in the GDM group were designated as NC when they were either not statistically different from those of controls or differed from controls by less than 10%.

<table>
<thead>
<tr>
<th>UniProt</th>
<th>Symbol</th>
<th>Entrez gene name</th>
<th>STZ Coverage (%)</th>
<th>Fold change</th>
<th>STZ + Insulin Coverage (%)</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>P16970</td>
<td>ABCD3</td>
<td>ATP-binding cassette, subfamily D, member 3</td>
<td>56.9</td>
<td>NC</td>
<td>61.6</td>
<td>-1.13*</td>
</tr>
<tr>
<td>Q80W57</td>
<td>ABCG2</td>
<td>ATP-binding cassette, subfamily G, member 2</td>
<td>-</td>
<td>-</td>
<td>33.8</td>
<td>-1.16*</td>
</tr>
<tr>
<td>Q07969</td>
<td>CD36</td>
<td>CD36 molecule (thrombospondin receptor)</td>
<td>41.1</td>
<td>-1.25**</td>
<td>31.8</td>
<td>-1.19*</td>
</tr>
<tr>
<td>P70500</td>
<td>CDIPT</td>
<td>CDP-diacylglycerol—inositol 3-phosphatidyltransferase</td>
<td>-</td>
<td>-</td>
<td>49.8</td>
<td>-1.19**</td>
</tr>
<tr>
<td>P04167</td>
<td>CYP2B6</td>
<td>Cytochrome P450, family 2, subfamily B, polypeptide 6</td>
<td>51.3</td>
<td>NC</td>
<td>40.3</td>
<td>-1.11*</td>
</tr>
<tr>
<td>P51869</td>
<td>CYP4F2</td>
<td>Cytochrome P450, family 4, subfamily F, polypeptide 2</td>
<td>56.3</td>
<td>NC</td>
<td>47.3</td>
<td>1.14**</td>
</tr>
<tr>
<td>Q9JJ46</td>
<td>EBP</td>
<td>Emopamil binding protein (sterol isomerase)</td>
<td>-</td>
<td>-</td>
<td>27.8</td>
<td>-1.25**</td>
</tr>
<tr>
<td>P35952</td>
<td>LDLR</td>
<td>Low density lipoprotein receptor</td>
<td>21.6</td>
<td>NC</td>
<td>22</td>
<td>1.23*</td>
</tr>
<tr>
<td>Q6AY20</td>
<td>M6PR</td>
<td>Mannose-6-phosphate receptor (cation dependent)</td>
<td>53.2</td>
<td>NC</td>
<td>41.4</td>
<td>1.19*</td>
</tr>
<tr>
<td>P46720</td>
<td>OATP1A1</td>
<td>Solute carrier organic anion transporter family, member 1A1</td>
<td>27.2</td>
<td>-1.14*</td>
<td>28.4</td>
<td>-1.17*</td>
</tr>
<tr>
<td>Q6RU5</td>
<td>RAC1</td>
<td>Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)</td>
<td>50</td>
<td>NC</td>
<td>24</td>
<td>1.27*</td>
</tr>
<tr>
<td>Accession</td>
<td>Gene ID</td>
<td>Description</td>
<td>Fold Ch</td>
<td>NC Ch</td>
<td>P Ch</td>
<td>Significance</td>
</tr>
<tr>
<td>-----------</td>
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<td>-------</td>
<td>-----</td>
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</tr>
<tr>
<td>Q9R0C9</td>
<td>SIGMAR1</td>
<td>Sigma non-opioid intracellular receptor 1</td>
<td>-</td>
<td>-</td>
<td>48</td>
<td>-1.17**</td>
</tr>
<tr>
<td>Q7TQM4</td>
<td>SOAT2</td>
<td>Sterol O-acyltransferase 2 (ACAT2)</td>
<td>37.4</td>
<td>NC</td>
<td>28.8</td>
<td>-1.2*</td>
</tr>
<tr>
<td>P22985</td>
<td>XDH</td>
<td>Xanthine dehydrogenase</td>
<td>27.1</td>
<td>NC</td>
<td>23.1</td>
<td>1.14**</td>
</tr>
</tbody>
</table>
Table 4. Comparisons of proteins that were altered in iTRAQ experiments to “Tox Lists” in the Ingenuity Knowledge Base. Datasets 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.

<table>
<thead>
<tr>
<th>“Tox List” ID (total number of list members)</th>
<th>STZ Upregulated</th>
<th>STZ Downregulated</th>
<th>STZ + Insulin Upregulated</th>
<th>STZ + Insulin Downregulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAR/RXR activation (28)</td>
<td>UGT1A1</td>
<td>-</td>
<td>-</td>
<td>CYP2B6</td>
</tr>
<tr>
<td>FXR/RXR activation (66)</td>
<td>ABCB4, APOA1, G6PC, SLC27A5</td>
<td>FASN</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LXR/RXR activation (55)</td>
<td>APOA1</td>
<td>ACACA, CD36, FASN</td>
<td>LDLR</td>
<td>CD36</td>
</tr>
<tr>
<td>Nrf2 activation (148)</td>
<td>CYP2A1, CYP4A11, CYP4A14</td>
<td>HMOX1</td>
<td>GSTA4, GSTM1, HSP90AA1</td>
<td>-</td>
</tr>
<tr>
<td>PPARα/RXR activation (118)</td>
<td>ACADL, APOA1</td>
<td>CD36, FASN</td>
<td>HSP90AA1</td>
<td>CD36</td>
</tr>
<tr>
<td>PXR/RXR activation (57)</td>
<td>ALDH1A2, G6PC, HMGCS2, UGT1A1</td>
<td>-</td>
<td>GSTM1</td>
<td>CYP2B6</td>
</tr>
<tr>
<td>Cytochrome P450 panel (rat) – substrate is a xenobiotic (23)</td>
<td>CYP2A1, CYP2D3</td>
<td>-</td>
<td>CYP2D3</td>
<td>CYP2B6</td>
</tr>
<tr>
<td>Xenobiotic metabolism (89)</td>
<td>CYP2A1, CYP2B9, CYP2D3, CYP51A1, UGT1A1</td>
<td>-</td>
<td>ALDH1L1, CYP2D3, CYP4F2, GSTA4, GSTM1</td>
<td>CYP2B6, UGT1A4</td>
</tr>
</tbody>
</table>
Figure 1

A

GLIB binding (% unbound)

Vehicle  GDM  Insulin-treated

B

SQV binding (% unbound)

Vehicle  GDM  Insulin-treated

C

GLIB binding (% unbound) vs SQV fu (% unbound)

$r^2 = 0.85$

$P < 0.0001$

D

GLIB binding (% unbound)

Vehicle  GDM  Insulin-treated

$#$
Figure 2

A: Non-treated diabetics (GDM)
   \( n = 625 \)

B: Insulin-treated diabetics
   \( n = 607 \)