Effects of glucose metabolism on the regulation of genes of fatty acid synthesis and triglyceride secretion in the liver

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Abstract Glucose disposal induces a signal that modulates the transcriptional regulation of genes involved in the glycolysis and lipogenesis pathways. To investigate the role of glucose metabolism on hepatic gene expression independently from insulin action, we overexpressed glucokinase, the limiting enzyme in the glycolysis pathway, in the liver of streptozotocin-induced type 1 diabetic rats. By microarray analysis, we observed that critical genes such as liver-type pyruvate kinase, malic enzyme, fatty acid synthase, and stearoyl-CoA desaturase 1 were enhanced multiple-fold, whereas genes involved in mitochondrial fatty acid oxidation and the Krebs cycle were downregulated. Despite the increase in expression of fatty acid synthesis genes and the presence of steatosis, no major alterations to the levels of genes involved in VLDL assembly and secretion, such as diacylglycerol acyltransferases 1 and 2 and microsomal triglyceride transfer protein, were observed. Overall, our data suggest that the gene expression pattern induced by glucose metabolism favors fatty acid storage in the liver rather than secretion into the circulation.

Supplementary key words gene expression • hepatic steatosis • glucokinase • microarray analysis

The increased intake of dietary carbohydrate in Western societies has elicited a great interest in unraveling the regulation of genes involved in de novo lipogenesis (DNL) in response to nutritional and hormonal signals. Enhanced activity of DNL enzymes has been shown to have an impact on the composition of triglycerides in the liver as well as on the composition of VLDL (1). Transcriptional regulation connects dietary signals with specific physiological responses. In recent years, it has become well established that glucose and insulin coordinate the transcriptional activation of gene expression in liver and that both are necessary for the activation to occur (2). Insulin enhances the lipogenic pathway by inducing expression of the transcription factor sterol-regulatory element binding protein 1c (SREBP-1c) (2, 3), a member of the basic domain helix-loop-helix leucine zipper family (4, 5). In addition to the transcriptional regulation of lipogenic gene expression, insulin has been implicated in the regulation of VLDL secretion by acutely inhibiting the incorporation of triglycerides into VLDL and redirecting them to the cytosol (6, 7). The hepatic transcription factor designated carbohydrate-responsive element binding protein (ChREBP) has been identified as a candidate for the induction of lipogenesis by glucose metabolism (8). ChREBP contains multiple domains, including a nuclear localization signal, polyproline, basic helix-loop-helix leucine zipper, and leucine zipper-like domains (8). ChREBP is localized in the cytoplasm under low-glucose conditions, and it translocates to the nucleus when glucose metabolism increases (9). There is some evidence suggesting that xylulose-5-phosphate, an intermediate of the pentose phosphate pathway, is the intracellular signaling compound by which excess carbohydrate activates ChREBP (10).

Glucose is converted to pyruvate via the glycolysis pathway and subsequently enters the Krebs cycle in mitochondria to be oxidized to CO₂ when ATP is required. When
abundant carbohydrate is available, glucose is converted to glycogen and fat, storage products that are used during fasting, strenuous exercise, or in a “fight-or-flight” situation (11). The conversion of carbohydrate to fatty acids involves enzymes such as ATP citrate lyase, acetyl-CoA carboxylase 1, and fatty acid synthase to generate palmitic acid (C16:0). Subsequent desaturation and/or elongation by stearoyl-CoA desaturase and long-chain fatty acyl elongase yields palmitoleic acid (C16:1), stearic acid (C18:0), and oleic acid (C18:1). The esterification of fatty acids to yield triglycerides and subsequent packaging into VLDL molecules involve several enzymes, and the details of this process are not yet completely understood. Diacylglycerol acyltransferases 1 and 2 (DGAT1 and DGAT2) are important for the esterification of diacylglycerol to yield triglycerides. It is believed that one of the two enzymes is cytoplasmic and mainly plays a role in the esterification of fatty acids that form the pool of triglycerides stored in the liver, whereas the second is present in the endoplasmic reticulum lumen and reesterifies fatty acids released from this pool to incorporate them into the triacylglycerol-rich particle that eventually forms the mature VLDL molecule (12–14). Microsomal triglyceride transfer protein (MTP) is also located in the lumen of the endoplasmic reticulum and is strictly necessary for the assembly and secretion of apolipoprotein B-containing lipoproteins (15, 16). Reduction of MTP activity levels by drug administration or in liver-specific knockout mice results in decreased levels of lipoproteins in plasma (17, 18). The impact of glucose metabolism on the regulation of all of these enzymes is not well understood. A deeper insight into the mechanisms by which carbohydrate controls gene expression may help in the design of better therapeutic treatments for diseases involving hepatic lipid metabolism, such as the metabolic syndrome and type 2 diabetes. The aim of this study was to investigate the role of glucose on the transcriptional regulation of genes involved in DNL and VLDL assembly in the liver.

MATERIALS AND METHODS

Animal groups

Athymic NIH nude rats (Cr: NIH-nu), 6 to 8 weeks old (100–130 g), were obtained from the National Cancer Institute (Frederick, MD). Guidelines for the use and care of laboratory animals at Mount Sinai School of Medicine and at Indiana University School of Medicine were followed. The animals were housed in a barrier facility during the course of the experiment and were kept under a 12 h light cycle (7:00 AM–7:00 PM). Rats were fed a standard chow diet. Animals were fasted overnight before intravenous administration of 80 mg/kg streptozotocin (STZ) dissolved in 100 mM citrate, pH 4.5, and 150 mM NaCl. Diabetic rats were selected based on blood glucose levels of >400 mg/dl and >10 g of body weight loss.

Microarray analysis

Four days after STZ administration, 9 \times 10^{11} viral particles (vp; 7.7 \times 10^{12} vp/kg) of the adenoviral vector Ad.EF1\text{a}GK (E1-deleted adenoviral vector containing an expression cassette with the glucokinase cDNA driven by the elongation factor 1\alpha promoter) or Ad.RSV\beta gal (expressing \beta-galactosidase from the Rous sarcoma virus promoter) (19, 20) was injected into the tail vein to groups of five rats. Reference data were collected from a group of nondiabetic rats. Rats were fed ad libitum and were euthanized under fed conditions on the morning of day 17. The liver was quickly removed and frozen on liquid nitrogen for RNA and protein isolation.

Hepatic triglyceride secretion

After STZ administration, animals received 5 \times 10^{11} vp (4.3 \times 10^{12} vp/kg) of Ad.EF1\text{a}GK or Ad.RSV\beta gal. STZ and nondiabetic control groups received vehicle. Triglyceride secretion rates were estimated on day 8 after virus administration. Rats were fasted for 4.5 h and given an intravenous bolus of tyloxapol (Triton WR-1339; Sigma Chemical Co., St. Louis, MO). Tyloxapol was dissolved in 0.9% NaCl and injected at a dose of 500 mg/kg body weight. Blood samples were collected from tail veins for the measurement of triglycerides at 0, 30, 60, and 90 min after tyloxapol injection. Triglyceride accumulation rates were determined as (mg/min) = \frac{1}{3}(TG_{t+1} - TG_t)/30 + (TG_{t+1} - TG_{t+2})/60 + (TG_{t+2} - TG_{t+3})/90 \times \text{plasma volume}, where TG_0, TG_30, TG_60, and TG_90 are triglyceride concentrations at 0, 30, 60, and 90 min, respectively. The plasma volume was estimated as 3.5% of body weight (21). Animals were euthanized under fed conditions on the morning of day 9, and livers were obtained for Oil Red O staining.

Blood glucose, serum insulin, NEFA, TG, and \beta-hydroxybutyrate measurement

Blood glucose was measured from a blood drop obtained from the tail vein using an Elite XL glucometer (Bayer, Elkhart, IN). Serum insulin levels were measured by RIA (Linco Research, St. Louis, MO) according to the manufacturer’s protocol. NEFAs were assayed using a kit from Wako (Richmond, VA). Triglycerides and \beta-hydroxybutyrate levels were measured by enzymatic assays using the GPO-Trinder and \beta-HBA (No. 310-UV) kits from Sigma Diagnostics (St. Louis, MO).

Microarray analysis

Total RNA was isolated from livers of rats that received Ad.EF1\text{a}GK or Ad.RSV\beta gal (five rats each) using Qiagen (Valencia, CA) Maxiprep kits. An additional purification step was carried out by precipitating RNA with lithium chloride. RNA was converted to double-stranded cDNA using the SuperScript Choice system for cDNA synthesis (Gibco BRL Life Technologies, Carlsbad, CA) and a T7-(dT)_{24} oligomer (Genset Corp., San Diego, CA). The double-stranded cDNA was transcribed in vitro with T7 polymerase to generate biotinylated copy RNA (Enzo BioArray HighYield RNA Transcript Labeling Kit; Enzo Life Sciences, Inc., Farmingdale, NY), which was subsequently purified with the RNeasy kit (Qiagen). The copy RNA was used to hybridize 10 independent Affymetrix Rat Genome U34A arrays (Affymetrix, Santa Clara, CA) using a rotary hybridization oven and postprocessed in Gene Chip Fluidics Station 400, according to the manufacturer’s protocol (Affymetrix). The array image was generated by a high-resolution GeneArray Scanner (Agilent, Palo Alto, CA). The U34A array contains ~7,000 full-length genes and 800 expressed sequence tags. Image files were analyzed with the application Microarray Analysis Suite version 5.0 (Affymetrix). Data were extracted after global scaling to 1,000. Probe sets showing a present call in at least half of the samples of at least one of the two groups were selected (22, 23), and a Welch’s t-test was performed to determine significant differences between the Ad.EF1\text{a}GK- and Ad.RSV\beta gal-treated groups. False discovery rate
Table 1. Primer sequences for real-time RT-PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Forward Primer (5′→3′)</th>
<th>Reverse Primer (5′→3′)</th>
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<td>ChREBP</td>
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<td>TGGCATACACTTGTGACG</td>
<td>TGGGCTGAGACCATCCAT</td>
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<td>DGAT1</td>
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<td>CGGTGATTACGTGATTG</td>
<td>GGCGCATCTGACATGGAAT</td>
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<td>DGAT2</td>
<td>NM_0010212345</td>
<td>ATCTCTCATGTCGACG</td>
<td>ACCCTCTGGAGGCTCTTC</td>
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<tr>
<td>EF2</td>
<td>NM_017245.2</td>
<td>GACGATCTCAGGTGACG</td>
<td>AATGATGTGTCGCAGCACCTC</td>
</tr>
<tr>
<td>FAS</td>
<td>M18767</td>
<td>TTTGCCAAGGAGGTGGGACG</td>
<td>TACCTCACAGAGATTGTCGG</td>
</tr>
<tr>
<td>FH</td>
<td>NM_017005</td>
<td>TGGCTGATTTGGTCAGGGAGGAC</td>
<td>TGGGATTTGAGTTTCTCTGGC</td>
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<td>FOXA2</td>
<td>NM_012743</td>
<td>TGAAGATGGAGGGCCAGAG</td>
<td>CCCACATATGGAGACATTT</td>
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<tr>
<td>FOXO1A</td>
<td>XM_342244</td>
<td>CTATCAGGTTGTGGACGAG</td>
<td>CCACTTCTGGAGCCACCA</td>
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<tr>
<td>HMG-CoA Red</td>
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<td>ACATCTCCACAGCACCTAC</td>
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<td>K-FABP</td>
<td>NM_145878</td>
<td>CCATGGCAGCCTTTAAGAG</td>
<td>ACCCTCTCATGAGCAGGTG</td>
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<tr>
<td>LacCer Syn</td>
<td>AB948687.1</td>
<td>TGGGCTGACGTGGGAGGAC</td>
<td>GTGAGCTGTCGAGCCCTCAT</td>
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<td>LDLR</td>
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<tr>
<td>L-PK</td>
<td>M11709</td>
<td>GATCATCTGCTGGAGAGGAC</td>
<td>GCAACCTGTCAGCAACATC</td>
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<tr>
<td>LRP</td>
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<td>TTCCTCGACATCGCAGACT</td>
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<td>SREBP-2a</td>
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<td>AATCGGCTCTTTTGGACACCA</td>
<td>TGTCGGCCCTCTCCCTTGGTT</td>
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</table>

ChREBP, carbohydrate-responsive element binding protein; DGAT, diacylglycerol acyltransferase; EF2, elongation factor 2; FAS, fatty acid synthase; FH, fumaryl hydratase 1; FOXO1A, forhead box 1A; HMG-CoA Red, 3-hydroxy-3-methylglutaryl coenzyme A reductase; K-FABP, keratinocyte fatty acid binding protein; LacCer Syn, lactosylceramide synthase; LDLR, low density lipoprotein receptor; L-PK, liver-type pyruvate kinase; LRP, LDL-related protein 1; MTP, microsomal triglyceride transfer protein; PGC, PPARγ coactivator; PPARγ, peroxisome proliferator-activated receptor γ; SREBP, sterol regulatory element binding protein.

Real-time PCR

Real-time PCR was used to quantifiable mRNA levels of the following genes: ChREBP, DGAT1, DGAT2, elongation factor 2 (EF2), FAS, forhead box A2 (FOXO2), forhead box O1A (FOXO1A), fumaryl hydratase 1 (FH), HMG-CoA reductase (HMG-CoA Red), keratinocyte fatty acid binding protein (K-FABP), lactosylceramide synthase, low density lipoprotein receptor (LDLR), liver-type pyruvate kinase, LDL receptor-related protein 1 (LRP), LDL receptor-related protein 1 (MTP), microsomal triglyceride transfer protein (MTP), PPARγ coactivator-1α (PPARγ), and SREBP.

Western blot

Approximately 50 mg of liver in 1 ml of lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin, and 1 µg/ml leupeptin) was completely homogenized with a tissue rotorator (IKA, Wilmington, NC) and incubated on ice for 30 min. Cellular debris was pelleted by centrifugation at 4°C. The fat layer was removed by aspiration, and the cleared supernatant was transferred to a fresh tube. Protein concentrations were determined by colorimetric assay (Bio-Rad, Hercules, CA). For Western blotting, 50 µg of liver proteins was run on an 18% or a 4–20% SDS-PAGE gel, transferred to a polyvinylidene difluoride membrane, and probed for K-FABP (R&D Systems, Minneapolis, MN), MTP (BD Biosciences, San Jose, CA), LDLR (Abcam, Cambridge, MA), or light chain of the LDLR-related protein 1 (Calbiochem, San Diego, CA). Membranes were then

Table 2. Fed serum parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ad.EF1aGK</th>
<th>Ad.RSVbGAL</th>
<th>PBS</th>
<th>Non-diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (ng/dl)</td>
<td>0.19 ± 0.12a</td>
<td>0.13 ± 0.02a</td>
<td>0.14 ± 0.03a</td>
<td>1.75 ± 0.82</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>226.8 ± 120.6b</td>
<td>575.4 ± 37.6</td>
<td>541.8 ± 114.6b</td>
<td>92.4 ± 5.0</td>
</tr>
<tr>
<td>β-Hydroxybutyrate (mg/dl)</td>
<td>8.7 ± 1.3c</td>
<td>14.1 ± 3.7</td>
<td>22.2 ± 5.6b</td>
<td>7.2 ± 5.0</td>
</tr>
<tr>
<td>FFA (mM)</td>
<td>0.78 ± 0.10c</td>
<td>0.76 ± 0.15a</td>
<td>0.94 ± 0.2a</td>
<td>0.48 ± 0.06</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>51.7 ± 15.8d</td>
<td>167.8 ± 81.9a</td>
<td>251.3 ± 33.5d</td>
<td>49.6 ± 16.2</td>
</tr>
</tbody>
</table>

*Significantly different from the nondiabetic group (P < 0.05).

Lipogenesis in response to glucose metabolism
TABLE 3. Distribution of probe sets significantly altered in glucokinase-overexpressing rats

<table>
<thead>
<tr>
<th>Functional Category</th>
<th>Percentage of Probe Sets</th>
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</thead>
<tbody>
<tr>
<td>Metabolism</td>
<td>28.5</td>
</tr>
<tr>
<td>Cellular physiological process</td>
<td>14.4</td>
</tr>
<tr>
<td>Cell communication</td>
<td>9.5</td>
</tr>
<tr>
<td>Organismal physiological process</td>
<td>6.2</td>
</tr>
<tr>
<td>Response to stimulus</td>
<td>6.0</td>
</tr>
<tr>
<td>Homeostasis</td>
<td>1.4</td>
</tr>
<tr>
<td>Morphogenesis</td>
<td>1.4</td>
</tr>
<tr>
<td>Cell differentiation</td>
<td>1.2</td>
</tr>
<tr>
<td>Regulation of cellular process</td>
<td>1.2</td>
</tr>
<tr>
<td>Death</td>
<td>1.0</td>
</tr>
<tr>
<td>Regulation of physiological process</td>
<td>0.4</td>
</tr>
<tr>
<td>Secretion</td>
<td>0.4</td>
</tr>
<tr>
<td>Coagulation</td>
<td>0.2</td>
</tr>
<tr>
<td>Pigmentation</td>
<td>0.2</td>
</tr>
<tr>
<td>Reproduction</td>
<td>0.2</td>
</tr>
<tr>
<td>Unclassified</td>
<td>53.6</td>
</tr>
</tbody>
</table>

RESULTS

Gene expression profiles

Glucokinase is the first and limiting enzyme of the glycolysis pathway, and its transcription is insulin-dependent. Given that glucose metabolism depends on the presence of insulin, it is difficult to determine the role of the former on the activation of gene expression in vivo. To determine the contribution of glucose metabolism to hepatic lipogenesis independently of insulin action, we overexpressed glucokinase in the liver of type 1 diabetic animals. Rats were rendered diabetic by intravenous administration of STZ at a dose of 80 mg/kg (20). We previously showed that STZ-induced diabetic rats do not have detectable levels of glucokinase in liver, as a result of the lack of insulin (20). Four days after STZ administration, rats received 9 × 10^11 vp of Ad.EF1αGK or Ad.RSVβgal (20). This vector dose resulted in ~90% liver transduction and an ~11-fold increase in glucokinase activity compared with nondiabetic animals (20). The equivalent volume of PBS was given to a group of STZ-treated rats, and a group of nondiabetic rats was used to collect reference data.

Seventeen days after vector administration, blood glucose was ~200 mg/dl in the group of rats that received the Ad.EF1αGK vector (~60% reduction) and >500 mg/dl in the groups that received PBS or the control vector.

TABLE 4. Genes involved in carbohydrate metabolism

<table>
<thead>
<tr>
<th>Affymetrix Identifier</th>
<th>Gene Name</th>
<th>Fold Change</th>
<th>P</th>
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<tr>
<td>Glycolysis</td>
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<tr>
<td>X05684_at</td>
<td>Pyruvate kinase, liver and red blood cells</td>
<td>6.02</td>
<td>&lt;0.01</td>
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<tr>
<td>U07181_g_at</td>
<td>Lactate dehydrogenase B</td>
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<td>&lt;0.01</td>
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<tr>
<td>X02291exion_s_at</td>
<td>Aldolase B</td>
<td>1.58</td>
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<tr>
<td>X02610_g_at</td>
<td>Enolase 1</td>
<td>1.48</td>
<td>&lt;0.05</td>
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<tr>
<td>Tricarboxylic acid cycle and associated reactions</td>
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<td>Pyruvate dehydrogenase kinase 1</td>
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<td>&lt;0.01</td>
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<td>AB010743_at</td>
<td>Uncoupling protein 2</td>
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<td>D10655_at</td>
<td>Dihydrolipoamide acetyltransferase</td>
<td>1.68</td>
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<td>U122968_at</td>
<td>Carbonic anhydrase 5</td>
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<td>U10357_at</td>
<td>Pyruvate dehydrogenase kinase 2</td>
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<td>D1324_s_at</td>
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<td>rc_AB10480_at</td>
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<td>L19927_at</td>
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<td>Pentose phosphate pathway</td>
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<td>Glycolipid biosynthesis</td>
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<td>Glucose-6-phosphatase, catalytic</td>
<td>1.73</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>K09243mRNA_s_at</td>
<td>Phosphoenolpyruvate carboxykinase 1</td>
<td>-1.78</td>
<td>&lt;0.05</td>
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</tbody>
</table>

Specifically stripped and reprobed with an anti-cyclophilin antibody (Novus Biologicals, Littleton, CO).

Statistical analysis

Data in the figures and Table 2 are expressed as means ± SD. Statistical analysis was performed using an unpaired two-tailed Student’s t-test. P < 0.05 was considered a significant difference.
Ad.RSVβgal (Table 2). Insulin levels were dramatically reduced in all STZ-treated animals (Table 2). To elucidate the gene expression pattern induced by glucose metabolism, RNA was obtained from liver of rats that received $9 \times 10^{11}$ vp/kg Ad.EF1αGK or Ad.RSVβgal, and Affymetrix GeneChip analysis was carried out using individual arrays for each animal. There were 984 probe sets that differed significantly between the two conditions ($P \leq 0.05$; FDR $\leq 0.17$), of which 483 were at $P \leq 0.01$ (FDR $\leq 0.070$) and 162 were at $P \leq 0.001$ (FDR $\leq 0.021$). Approximately

### TABLE 5. Genes involved in lipid metabolism

<table>
<thead>
<tr>
<th>Affymetrix Identifier</th>
<th>Gene Name</th>
<th>Fold Change</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>L13039_s_at</td>
<td>Calpain I heavy chain</td>
<td>6.05</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>U3880U1TR1_g_at</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase 2</td>
<td>5.31</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>D42137exon_s_at</td>
<td>Annexin A5</td>
<td>3.07</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>rc_AA893191_g_at</td>
<td>Phosphatidic acid phosphatase 2c</td>
<td>1.73</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>U39572_s_at</td>
<td>Phosphatidylinositol 4-kinase</td>
<td>1.57</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>L07736_at</td>
<td>Carnitine palmitoyltransferase 1, liver</td>
<td>1.64</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>D16479_at</td>
<td>Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase</td>
<td>1.25</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>U08976_s_at</td>
<td>Enoyl-CoA hydratase 1</td>
<td>1.57</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>rc_AI171506_s_at</td>
<td>Malic enzyme</td>
<td>5.31</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>J02791_at</td>
<td>Acetyl-CoA dehydrogenase, medium chain</td>
<td>4.2</td>
<td>&lt;0.01</td>
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<tr>
<td>L03294_at</td>
<td>Lipoprotein lipase</td>
<td>2.56</td>
<td>&lt;0.01</td>
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<td>rc_AA893242_g_at</td>
<td>Fatty acid CoA ligase, long chain 2</td>
<td>2.93</td>
<td>&lt;0.01</td>
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<tr>
<td>Y17295cds_s_at</td>
<td>Peroxiredoxin 6</td>
<td>2.30</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>S81497_i_at</td>
<td>Lipase A, lysosomal acid</td>
<td>1.37</td>
<td>&lt;0.05</td>
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<tr>
<td>rc_AI169372_g_at</td>
<td>Arachidonic acid epoxygenase</td>
<td>1.36</td>
<td>&lt;0.05</td>
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<tr>
<td>M81225_s_at</td>
<td>Farnesyltransferase, CAAX box, α</td>
<td>1.29</td>
<td>&lt;0.05</td>
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<tr>
<td>S69874_s_at</td>
<td>Fatty acid binding protein 5, epidermal</td>
<td>8.07</td>
<td>&lt;0.01</td>
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<tr>
<td>S85100_at</td>
<td>Solute carrier family 27 (fatty acid transporter), member 32</td>
<td>1.54</td>
<td>&lt;0.01</td>
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<td>K03045cds_s_at</td>
<td>Retinol binding protein 4, plasma</td>
<td>1.50</td>
<td>&lt;0.05</td>
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<td>rc_AA895242_g_at</td>
<td>Fatty acid CoA ligase, long chain 2</td>
<td>1.65</td>
<td>&lt;0.05</td>
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<tr>
<td>Y17295cds_s_at</td>
<td>Peroxiredoxin 6</td>
<td>1.73</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>S81497_i_at</td>
<td>Lipase A, lysosomal acid</td>
<td>1.37</td>
<td>&lt;0.05</td>
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<tr>
<td>rc_AI236284_s_at</td>
<td>Fatty acid CoA ligase, long chain 3</td>
<td>1.63</td>
<td>&lt;0.05</td>
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<tr>
<td>L34049_g_at</td>
<td>Low density lipoprotein receptor-related protein 2</td>
<td>1.77</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>X04979_at</td>
<td>Apolipoprotein E</td>
<td>1.61</td>
<td>&lt;0.05</td>
</tr>
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<td>rc_J0596cds_g_at</td>
<td>Apolipoprotein C-III</td>
<td>1.53</td>
<td>&lt;0.01</td>
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<td>M00001_1_at</td>
<td>Apolipoprotein A-I</td>
<td>2.06</td>
<td>&lt;0.01</td>
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<tr>
<td>X54096_at</td>
<td>Lecithin:cholesterol acyltransferase</td>
<td>1.57</td>
<td>&lt;0.01</td>
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<td>rc_AA889218_at</td>
<td>Apolipoprotein M</td>
<td>1.56</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>L12380_at</td>
<td>ADP-ribosylation factor 1</td>
<td>1.54</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>M21208mRNA_s_at</td>
<td>Cytochrome P450, subfamily 17</td>
<td>2.83</td>
<td>&lt;0.01</td>
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<tr>
<td>S88431s_s_at</td>
<td>Aldo-keto reductase family 1, member D1 (Δ4-3-ketosteroid-5β-reductase)</td>
<td>3.04</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>U08976_at</td>
<td>α-Methylacyl-CoA racemase</td>
<td>1.99</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>D43964_at</td>
<td>Bile acid-CoAminoo acid-Nacyltransferase</td>
<td>1.57</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>
28.5% of the significant probe sets ($P \leq 0.05$) were involved in metabolism and 14.4% were involved in cellular physiological processes (Table 3). All genes significantly changed are listed in supplementary appendix I (available online).

Classification using biological function was performed to determine alterations to carbohydrate and lipid metabolism pathways (Tables 4, 5). mRNA levels of critical genes were confirmed by real-time RT-PCR (Fig. 1). Genes involved in glycolysis, such as lactate dehydrogenase and liver-type pyruvate kinase, were upregulated (Table 4, Fig. 1), whereas genes involved in the tricarboxylic acid cycle (Krebs cycle) and oxidative phosphorylation, such as FH and ATP synthase, respectively, were downregulated (Table 4). This suggests that conversion of pyruvate to CO$_2$ was not the primary pathway in the liver of the glucokinase-overexpressing animals. Levels of mRNA of the lipogenic genes fatty acid synthase, ATP citrate lyase, and malic enzyme were increased multiple-fold (Table 5, Fig. 1), indicating that the de novo fatty acid synthesis pathway was upregulated in Ad.EF1αGK-treated animals. Consistent with this observation, several genes of the pentose phosphate pathway, including glucose 6-phosphate dehydrogenase, transaldolase, and transketolase, were also increased. The conversion of glucose to fatty acids requires NADPH for the addition of malonyl units into the nascent acyl-ACP chain. Approximately 60% of the NADPH is produced through the cascade of reactions of the pentose phosphate pathway, whereas the pyruvate/malate cycle generates ~40% (11). The fructose transporter, GLUT5, was upregulated by 4.19-fold, and the expression of several genes involved in glycoprotein biosynthesis was also increased (Table 4).

In addition to enhanced de novo production of fatty acids from glucose, we observed that glucokinase overexpression induced alterations to other genes of fatty acid metabolism. In particular, stearoyl-coenzyme A desaturase 1 (SCD-1), the limiting enzyme in the biosynthesis of monounsaturated fatty acids (mainly oleate), was substantially upregulated (154-fold; Table 5). The mRNA of SCD-2 was also enhanced by 3.1-fold. Oleate is necessary for the production of triglycerides, phospholipids, and cholesterol esters and is also an important mediator of signal transduction, among other functions (26).

Carnitine-Palmitoyl Transferase 1 (CPT1) is the transporter of long-chain fatty acids into mitochondria for oxidation to acetyl-CoA. In contrast to the increase in fatty acid synthesis gene expression, mRNA levels of CPT1 were reduced in glucokinase-treated rats (Table 5). In addition, mitochondrial genes of the β-oxidation pathway, such as the trifunctional protein, acetyl-CoA dehydrogenase, and enoyl-CoA hydratase, as well as genes involved in peroxisomal fatty acid oxidation were downregulated (Table 5). The levels of β-hydroxybutyrate in rats treated with the Ad.EF1αGK vector were lower than in control rats treated with the Ad.RSVβgal vector and similar to the levels observed in normal rats (Table 2), reflecting what would be expected from the gene expression pattern. Thus, glucokinase overexpression induced de novo fatty acid synthesis and a reduction of fatty acid oxidation.

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Fig. 1. RNA abundance based on real-time RT-PCR analysis. FABP5, fatty acid binding protein 5; FAS, fatty acid synthase; FH, fumarate hydratase; HMG-CoA Red, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LacCer Syn, lactosylceramide synthase; L-PK, liver-type pyruvate kinase; STZ, streptozotocin. The inset in the FABP5 graph shows Western blot analysis of FABP5. GK, rats treated with Ad.EF1αGK; βgal, rats treated with Ad.RSVβgal; Norm., normal rats. Data shown are expressed as means ± SD. * $P < 0.05$ between nondiabetic and Ad.RSVβgal-treated diabetic rats; ** $P < 0.05$ between Ad.RSVβgal- and Ad.EF1αGK-treated rats. Numbers within boxes represent the fold expression level in Ad.EF1αGK-treated rats with respect to Ad.RSVβgal-treated rats.
Gene expression analysis of genes involved in triglyceride secretion

We were surprised by the fact that the increase in mRNA levels of enzymes involved in fatty acid biosynthesis was not associated with an increase in the levels of genes necessary for triglyceride packaging into VLDL and secretion. Based on the microarray data, no difference was observed between Ad.EF1αGK- and Ad.RSVβgal-treated animals for enzymes such as DGAT1 and apolipoprotein B. To obtain a deeper insight into the mRNA levels of genes critical in the secretory pathway, we analyzed levels of DGAT1, DGAT2, and MTP by real-time RT-PCR (Fig. 2). We observed that DGAT1 and DGAT2 were regulated in an opposite way in type 1 diabetes rats compared with normal animals: whereas DGAT1 mRNA levels increased in type 1 diabetes animals, DGAT2 levels decreased. Interestingly, no alterations to the mRNA levels of DGAT2 were observed in the Ad.EF1αGK-treated rats (Fig. 2), and only a small difference was seen for DGAT1 (P < 0.05), suggesting that increased de novo fatty acid synthesis has little impact on the transcription of genes of the secretory pathway. No alterations to the levels of MTP mRNA or protein were observed between normal and type 1 diabetes animals or between Ad.EF1αGK- and Ad.RSVβgal-treated rats (Fig. 2). This gene expression pattern favoring fatty acid synthesis in liver was associated with the presence of hepatic steatosis (Fig. 3B) (20). Interestingly, circulating levels of triglycerides were significantly lower in the animals treated with the Ad.EF1αGK adenovirus than in rats treated with the control virus Ad.RSVβgal, suggesting the possibility of increased uptake of chylomicron remnants and/or VLDL by the liver. Thus, we analyzed mRNA levels of LDLR and LDLR-related protein 1. No alterations in gene expression or protein levels were observed in the Ad.EF1αGK-treated animals compared with Ad.RSVβgal-treated controls (Fig. 2).

Hepatic triglyceride secretion

To further assess the contribution of glucose metabolism to the induction of triglyceride-rich lipoprotein secretion, we determined serum triglyceride concentrations after tyloxapol (Triton WR-1339) administration. Tyloxapol is a nonionic detergent that inhibits lipoprotein lipase and prevents triglyceride uptake by tissues. The secretion rate in diabetic vehicle-treated animals was lower than in nondiabetic controls (Fig. 3A), confirming previously published data (27, 28). Rats that received the Ad.RSVβgal vector had significantly lower secretion levels compared with STZ vehicle-treated animals. We do not know whether the adenovirus or expression of the transgene induced the reduction in secretion in the Ad.RSVβgal-treated rats. It has been shown previously that adenovirus-mediated LacZ expression in human hepatocytes results in a mild increase of glycolysis (29). Expression of glucokinase significantly increased triglyceride

Fig. 2. RNA and protein abundance of genes involved in VLDL assembly and secretion. A: Real-time RT-PCR analysis. B: Western blot analysis. DGAT, diacylglycerol acyltransferase; LDLR, low density lipoprotein receptor; LRP, LDLR-related protein 1; MTP, microsomal transfer protein. Data shown are expressed as means ± SD. * P < 0.05 compared with the nondiabetic group; ** P < 0.05 between Ad.RSVβgal-treated rats and Ad.EF1αGK-treated rats.
secretion compared with Ad.RSVβgal-treated rats, but the rate of secretion was not sufficient to prevent the development of hepatic steatosis (Fig. 3A, B).

Gene expression analysis of transcription factors/coactivators relevant to glucose and lipid metabolism

Finally, we measured mRNA levels of the transcription factors/coactivators important for glucose and fatty acid metabolism (Fig. 4). There was a substantial decrease in SREBP-1 mRNA levels between nondiabetic animals and diabetic animals (Fig. 4), consistent with the fact that expression of this transcription factor is regulated by insulin. A small increase in SREBP-1 was observed in the animals treated with Ad.EF1αGK compared with the control vector, Ad.RSVβgal (Fig. 4), which had not been detected by microarray analysis. Given that the glucokinase-overexpressing animals had insulin levels similar to those of the Ad.RSVβgal control animals, the increase is likely to be insulin-independent. Miyazaki and colleagues (30) have shown that oleate produced by stearoyl-CoA desaturase is necessary for fructose-mediated induction of lipogenic gene expression by SREBP-1c. Given that SCD-1 was enhanced multiple-fold in the Ad.EF1αGK-treated rats, it is possible that the oleate produced led to an increase in SREBP-1c mRNA. We also measured the levels of another member of the same family, SREBP-2, which mainly regulates the transcription of genes involved in cholesterol synthesis, and observed that its mRNA levels were not increased in the animals that received the Ad.EF1αGK adenoviral vector (Fig. 4) (this gene was not represented in the Affymetrix array). Expression levels of a SREBP-2 target (31), HMG-CoA Red (a limiting enzyme of the cholesterol synthesis pathway), were not found to be upregulated or downregulated by real-time RT-PCR, consistent with the microarray analysis results (Fig. 1). ChREBP mRNA levels were significantly higher in livers of rats overexpressing glucokinase than in livers of rats that received the control adenovirus, Ad.RSVβgal (1.8-fold; Fig. 4). Analyses of PGC-1β, FOXO1A, and FOXA2 were not altered under diabetic conditions or upon overexpression of glucokinase. With regard to PGC-1α, it was increased in STZ-treated rats compared with normal animals, and glucokinase overexpression had a minor effect on the expression of this gene but was not significantly different from the Ad.RSVβgal-treated group (Fig. 4). PPARγ, a prolipogenic nuclear receptor, was downregulated in Ad.RSVβgal-treated diabetic animals compared with normal rats and significantly upregulated by glucose metabolism in glucokinase-expressing animals (Fig. 4).

DISCUSSION

Mammals have evolved to adapt their metabolism to the nutritional environment. When abundant food supplies are accessible, nutrients are stored for subsequent use during periods of food shortage. To ensure that dietary carbohydrates are either used to produce energy or stored in the form of lipids, multiple genes of the glycolysis as well as the lipogenic pathways are regulated at the transcriptional and posttranscriptional levels. Because glucose is constantly required at a high rate by multiple tissues, mammals have evolved to sense glucose levels and adapt
the expression of genes to glucose availability. Concomitant increases in insulin and glucose levels after a meal preclude individual analyses of their roles in the regulation of gene expression. Glucokinase is the first and limiting enzyme in the glycolytic pathway, and transcription of its gene is activated by insulin (32). Dentin and colleagues (33) have shown that in the absence of glucokinase, glycolytic and lipogenic genes are not induced upon refeeding mice a high-carbohydrate diet. In our study, we generated an animal model of type 1 diabetes with enhanced glucose disposal as a result of glucokinase overexpression, thereby bypassing the insulin-dependent step in glycolysis. We show in this study that glucose disposal is sufficient to activate the transcription of key genes of the glycolysis, pentose phosphate, and lipogenesis pathways. This increase was accompanied by downregulation of a large number of genes involved in fatty acid oxidation in mitochondria, which correlated with a decrease in ketone levels in the serum of the animals. Thus, glucokinase overexpression resulted in the upregulation of lipogenesis and the reduction of fatty acid oxidation (Fig. 5).

We anticipated that increased transcription of lipogenic genes would be associated with an increase in the expression of genes necessary for the assembly and secretion of VLDL. However, our data indicate that mRNA levels of DGAT1 and DGAT2 change in response to insulin levels (DGAT1 was increased in type 1 diabetes compared with normal animals and DGAT2 was decreased), and only DGAT1 is minimally affected by overexpression of glucokinase (Fig. 2). Thus, despite the fact that DGAT 1 and DGAT2 lie downstream of de novo fatty acid synthesis, glucose metabolism does not have a major effect on the transcription of these two genes. The enzyme MTP, critical for the assembly of VLDL, was not upregulated or downregulated in STZ-treated rats compared with normal control rats. Furthermore, we did not observe differences in the level of expression between rats treated with the Ad.EF1α-GK or Ad.RSVβgal adenovirus (Fig. 2). Transcription of MTP has been shown to correlate strongly with protein and activity levels (34, 35). Cell culture studies have suggested that MTP gene expression is positively regulated by glucose in primary hepatocytes (36) and negatively regulated by insulin and glucose in HepG2 cells (37). Another study has shown that SREBPs negatively regulate MTP by binding to SRE elements in the promoter (38). In vivo, MTP levels and activity remained unchanged in livers of type 1 diabetic rats, suggesting that insulin does not regulate MTP (39). Our data further support this observation and indicate that in vivo, glucose metabolism does not have an impact on the expression of MTP either.
Together, these studies suggest that the transcriptional control of MTP is complex and may be subjected to multiple positive and negative factors.

The fact that genes such as DGAT1, DGAT2, and MTP were not altered in glucokinase-overexpressing animals, together with the presence of hepatic steatosis, suggest that the fate of fatty acids synthesized de novo is to be stored in the liver rather than to be secreted immediately. This hypothesis is in agreement with evidence indicating that the bulk of fatty acids incorporated into VLDL come from a pool of triglycerides stored in the liver rather than to be secreted immediately. The availability of isotopic methods that measure the incorporation of \[^{13}C\]acetate or deuterated water into fatty acids has allowed more accurate determinations of DNL in humans. Donnelly and colleagues (1) have shown that the contribution of DNL to intrahepatic fat is <5% in healthy subjects but increases to 26% in individuals with nonalcoholic fatty liver disease. It has also been shown that human de novo fatty acid synthesis is stimulated by a eucaloric low-fat/high-carbohydrate diet (48) and that lean as well as obese individuals fed a low-fat/high-carbohydrate diet have increased levels of DNL compared with subjects fed a high-fat/low-carbohydrate diet (49). Although we have not determined enzyme activities and we cannot discount possible posttranslational regulation by glucose metabolism, our data suggest that the physiological response induced by glucose clearly promotes an increase in the pathways that convert glucose to fatty acids without having a major effect on genes involved in their secretion, which may partially explain why carbohydrate-rich diets induce hepatic steatosis.

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REFERENCES


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