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.—Morral, N., H. J. Edenberg, S. R. Witting, J. Altomonte, T. Chu, and M. Brown. **Effects of glucose metabolism on the regulation of genes of fatty acid synthesis and triglyceride secretion in the liver. *J. Lipid Res.* 2007.** 48: **1499–1510.**

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 CO_2 when ATP is required. When

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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-rnu), 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, 9×10^{11} viral particles (vp; 7.7×10^{12} vp/kg) of the adenoviral vector Ad.EF1αGK (E1-deleted adenoviral vector containing an expression cassette with the glucokinase cDNA driven by the elongation factor 1α promoter) or Ad.RSVβgal (expressing β-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 5×10^{11} vp (4.3×10^{12} vp/kg) of Ad.EF1αGK or Ad.RSVβgal. 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 300 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): $1/3[(TG_{30} - TG_0)/30 + (TG_{60} - TG_0)/60 + (TG_{90} - TG_0)/90] \times$ 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 β-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 β-hydroxybutyrate levels were measured by enzymatic assays using the GPO-Trinder and β-HBA (No. 310-UV) kits from Sigma Diagnostics (St. Louis, MO).

Microarray analysis

Total RNA was isolated from livers of rats that received Ad.EF1αGK or Ad.RSVβgal (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 post-processed 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.EF1αGK- and Ad.RSVβgal-treated groups. False discovery rate

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TABLE 1. Primer sequences for real-time RT-PCR analysis

Gene	Accession Number	Forward Primer (5′→3′)	Reverse Primer (5′→3′)
ChREBP	AB074517	TGCCATCAACTTGTGCCAGC	TGCGGTAGACACCATCCCAT
DGAT1	NM_053437	CCGTGGTATCCTGAATTGGT	GGCGCTTCTCAATCTGAAAT
DGAT2	NM_001012345	ATCTTCTCTGTCACCTGGCT	ACCTTTCTTGGGCGTGTTCC
EF2	NM_017245.2	GACCAGTTCCTTGTGAAGACCG	AATGATGTGCTCCCCAGACTCC
FAS	M76767	TTTGCCAAGGAGGTGCGAAC	TACTCAGCAGAAGATGTGCGG
FH	NM_017005	GTGCTGTATTGTCAGGGGAAGC	TGGGATTGGCATTCTCTCCGTC
FOXA2	NM_012743	TGAAGATGGAAGGGCACGAG	CCCACATAGGATGACATGTTC
FOXO1A	XM_342244	TACTTCAAGGATAAGGGCGACA	TTTTCTTAGCAGCCCGTCCTC
HMG-CoA Red	NM_013134.2	CAGCACTGTCGTCATTCATTTCC	ACATTCCACCAGAGCGTCAAGG
K-FABP	NM_145878	CCATGGCCAGCCTTAAGGA	ACCTTCTCATAGACCCGAGT
LacCer Syn	AF048687.1	TCGGAACTATTACGGATGCGG	GTGAACTCTGTTCCAAAGGTCG
LDLR	NM_175762.2	CCGCCTCTATTGGGTTGATTC	GTTGCCTCACACCAGTTTACC
L-PK	M11709	GTATCATGCTGTCCGGAGAGAC	GCCAACCTGTCACCACAATCAC
LRP	XM_243524	TACGCCACCAACTCAGACAACG	TTTCCCGTCACTTCCCAGACTG
MTP	XM_227765	GAACCTGAGAACCTGTCCAACG	TGAACTTGCTAAGGAGGGCTTG
PGC-1α	NM_031347	TGAATGACCTGGACACAGACA	ATCAAATGAGGGCAATCCGTC
PGC-1β	AY_188951	TCCCCAGTGTCTGAAGTGGA	TTCTTGTCCTGGGTGCCATC
PPARγ	AB019561	GGTGTGATCTTAACTGTCGG	TTCAGCTGGTCGATATCACT
SREBP-1	XM_213329	GGTCACCGTTTCTTCGTGGATG	GGCTGAGCGATACAGTTCAATGC
SREBP-2	XM_216989	ATTCCCTTGTTTTGACCACGC	TGTCCGCCTCTCTCCTTCTTTG

ChREBP, carbohydrate-responsive element binding protein; DGAT, diacylglycerol acyltransferase; EF2, elongation factor 2; FAS, fatty acid synthase; FH, fumarate hydratase 1; FOXA2, forkhead box A2; FOXO1A, forkhead box O1A; 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, LDLR-related protein 1; MTP, microsomal triglyceride transfer protein; PGC, PPARγ coactivator; PPARγ, peroxisome proliferator-activated receptor γ; SREBP, sterol-regulatory element binding protein.

(FDR) was calculated according to Benjamini and Hochberg (24). Gene probes that resulted in statistically significant differences ($P < 0.05$) were loaded onto the NIH-DAVID database (25) for analysis of gene ontologies according to the biological process.

Real-time PCR

Real-time PCR was used to quantify mRNA levels of the following genes: ChREBP, DGAT1, DGAT2, elongation factor 2 (EF2), FAS, forkhead box A2 (FOXA2), forkhead box O1A (FOXO1A), fumarate 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, MTP, peroxisome proliferator-activated receptor γ (PPARγ); PPARγ coactivator-1α and -1β (PGC-1α and PGC-1β), SREBP-1, and SREBP-2. Primers used for amplification are listed in **Table 1**. Real-time PCR was performed using an ABI PRISM 7700 instrument (ABI, Foster City, CA) and the SYBR Green Qiagen One-Step RT-PCR kit (Qiagen) according to the manufacturer's protocol and using 0.5 μM of each primer. Primer pairs were designed to amplify a fragment of 103–450 bp and were first tested to yield a single PCR product based on the melting curve and confirmation by agarose gel electrophoresis. A standard curve was generated with serial dilutions of an RNA sample from a normal rat. Quantification of mRNA levels in test samples was measured by analyzing 50 ng of total RNA in triplicate and comparing threshold cycle values with those of the standard curve. Because β-actin was found to be expressed at significantly different levels between the treatment groups, the eukaryotic translation EF2 gene was used as a loading control. Values were expressed relative to the nondiabetic group.

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 rotor-stator (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

Parameter	Ad.EF1αGK	Ad.RSVβgal	PBS	Nondiabetic
Insulin (ng/dl)	0.19 ± 0.12[a]	0.13 ± 0.02[a]	0.14 ± 0.03[a]	1.75 ± 0.82
Glucose (mg/dl)	226.8 ± 120.6[b]	575.4 ± 37.6[a]	541.8 ± 114.6[a]	92.4 ± 5.0
β-Hydroxybutyrate (mg/dl)	8.7 ± 1.3[b]	14.1 ± 3.7	22.2 ± 5.6[a]	7.2 ± 5.0
FFA (mM)	0.78 ± 0.10[a]	0.76 ± 0.15[a]	0.94 ± 0.2[a]	0.48 ± 0.06
Triglycerides (mg/dl)	51.7 ± 15.8[b]	167.8 ± 81.9[a]	251.3 ± 33.5[a]	49.6 ± 16.2

[a] Significantly different from the nondiabetic group ($P < 0.05$).
[b] Significantly different from the Ad.RSVβgal and PBS groups ($P < 0.05$).

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TABLE 3. Distribution of probe sets significantly altered in glucokinase-overexpressing rats

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

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.

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

Affymetrix Identifier	Gene Name	Fold Change	*P*
Glycolysis			
X05684_at	Pyruvate kinase, liver and red blood cells	6.02	<0.01
U07181_g_at	Lactate dehydrogenase B	2.48	<0.01
X02291exon_s_at	Aldolase B	1.58	<0.05
X02610_g_at	Enolase 1α	1.48	<0.05
Tricarboxylic acid cycle and associated reactions			
L22294_at	Pyruvate dehydrogenase kinase 1	2.14	<0.01
AB010743_at	Uncoupling protein 2	1.7	<0.05
D10655_at	Dihydrolipoamide acetyltransferase	1.68	<0.01
rc_AI171734_s_at	Fumarate hydratase 1	−3.11	<0.01
U12268_at	Carbonic anhydrase 5	−3.09	<0.01
U10357_at	Pyruvate dehydrogenase kinase 2	−1.78	<0.05
D13124_s_at	ATP synthase, H+-transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 2	−1.41	<0.05
D13123_s_at	ATP synthase, H+-transporting, mitochondrial F0 complex, subunit c, isoform 1	−1.36	<0.05
rc_AI010480_at	Malate dehydrogenase, mitochondrial	−1.32	<0.05
L19927_at	ATP synthase, H+-transporting, mitochondrial F1 complex, γ polypeptide 1	−1.27	<0.05
X56133_at	Mitochondrial H+-ATP synthase α subunit	−1.24	<0.05
Pentose phosphate pathway			
U09256_at	Transketolase	2.56	<0.01
rc_AA799452_at	Transaldolase 1	1.48	<0.01
X07467_at	Glucose-6-phosphate dehydrogenase	1.41	<0.05
Carbohydrate transport			
D13871_s_at	Solute carrier family 2, member 5 (GLUT5)	4.19	<0.01
Glycoprotein biosynthesis			
U21662_at	Mannosyl (α-1,6-)-glycoprotein β-1,2-*N*-acetylglucosaminyltransferase	1.81	<0.01
D10261_g_at	α-2-HS-glycoprotein	1.61	<0.01
D16302_at	*N*-Acetylglucosaminyltransferase I	1.28	<0.01
AF087431_g_at	Glucosidase 1	1.27	<0.05
Glycogen metabolism			
U96130_at	Glycogenin	2.33	<0.01
rc_AA800190_g_at	Brain glycogen phosphorylase	1.45	<0.05
X73653_at	Glycogen synthase kinase 3β	1.37	<0.05
L11694_at	Phosphoglucomutase 1	−2.03	<0.01
Gluconeogenesis			
L37333_s_at	Glucose-6-phosphatase, catalytic	1.73	<0.05
K03243mRNA_s_at	Phosphoenolpyruvate carboxykinase 1	−1.78	<0.05

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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 9×10^{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 ($P \leqslant 0.05$; FDR $\leqslant$ 0.17), of which 483 were at $P \leqslant 0.01$ (FDR $\leqslant$ 0.070) and 162 were at $P \leqslant 0.001$ (FDR $\leqslant$ 0.021). Approximately

TABLE 5. Genes involved in lipid metabolism

Affymetrix Identifier	Gene Name	Fold Change	P
Fatty acid biosynthesis			
J02585_at	Stearoyl-CoA desaturase 1	154	<0.01
rc_AI171506_g_at	Malic enzyme 1	5.3	<0.01
J05210_g_at	ATP citrate lyase	4.2	<0.01
M76767_s_at	Fatty acid synthase	3.57	<0.01
rc_AA875269_at	Stearoyl-CoA desaturase 2	3.12	<0.01
Fatty acid oxidation, mitochondrial			
X15958_at	Enoyl-CoA hydratase, short chain 1	−2.16	<0.01
J02791_at	Acetyl-CoA dehydrogenase, medium chain	−1.84	<0.01
L07736_at	Carnitine palmitoyltransferase 1, liver	−1.64	<0.05
D16479_at	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), β subunit	−1.25	<0.01
Fatty acid oxidation, peroxisomal			
J02749_at	Acetyl-CoA acyltransferase 1 (peroxisomal 3-oxoacyl-CoA thiolase)	2.41	<0.01
rc_AI101743_s_at	Peroxisomal multifunctional enzyme type II	−2.93	<0.05
rc_AA799489_g_at	Acyl-CoA oxidase	−1.83	<0.05
U08976_at	Enoyl-CoA hydratase 1	−1.57	<0.05
Acylglycerol and phospholipid metabolism			
L13039_s_at	Calpactin I heavy chain	6.05	<0.01
U83880UTR#1_g_at	Glycerol-3-phosphate dehydrogenase 2	5.31	<0.01
D42137exon_s_at	Annexin A5	3.07	<0.01
rc_AA893191_g_at	Phosphatidic acid phosphatase type 2c	1.73	<0.05
U39572_s_at	Phosphatidylinositol 4-kinase	1.57	<0.05
L06096_g_at	Inositol 1,4,5-triphosphate receptor 3	1.52	<0.05
S57478cds_s_at	Annexin 1	1.43	<0.01
rc_AA891107_at	Diphosphoinositol polyphosphate phosphohydrolase type II	−1.28	<0.05
Glycosphingolipid metabolism			
AF048687_s_at	UDP-Gal:βGlcNAc β-1,4-galactosyltransferase, polypeptide 6	6.04	<0.01
AF047707_at	UDP-glucose:ceramide glycosyltransferase	1.35	<0.05
Arachidonic acid metabolism and secretion			
rc_AI169372_g_at	Arachidonic acid epoxygenase	1.36	<0.05
Protein lipidation			
M81225_at	Farnesyltransferase, CAAX box, α	1.29	<0.05
Lipid transport			
S69874_s_at	Fatty acid binding protein 5, epidermal	8.07	<0.01
M58287_s_at	Sterol carrier protein 2	−1.87	<0.05
D85100_at	Solute carrier family 27 (fatty acid transporter), member 32	−1.54	<0.01
K03045cds_s_at	Retinol binding protein 4, plasma	−1.50	<0.05
Regulation of fatty acid metabolism			
AB010428_s_at	Cytosolic acyl-CoA thioesterase 1	8.97	<0.01
L03294_at	Lipoprotein lipase	2.56	<0.01
D30666_at	Fatty acid CoA ligase, long chain 3	1.63	<0.05
rc_AI236284_s_at	Fatty acid CoA ligase, long chain 4	−1.9	<0.01
rc_AA893242_g_at	Fatty acid CoA ligase, long chain 2	−1.65	<0.05
Y17295cds_s_at	Peroxiredoxin 6	−1.73	<0.01
S81497_i_at	Lipase A, lysosomal acid	−1.37	<0.05
Cholesterol metabolism			
L07114_at	Apolipoprotein B editing complex 1	1.92	<0.05
L34049_g_at	Low density lipoprotein receptor-related protein 2	1.77	<0.01
X04979_at	Apolipoprotein E	1.61	<0.05
J02596cds_g_at	Apolipoprotein C-III	1.53	<0.05
M00001_i_at	Apolipoprotein A-I	−2.06	<0.01
X54096_at	Lecithin:cholesterol acyltransferase	−1.81	<0.01
rc_H33491_at	Phenylalkylamine Ca^2+ antagonist (emopamil) binding protein	−1.66	<0.05
rc_AA893213_at	Apolipoprotein M	−1.56	<0.01
L12380_at	ADP-ribosylation factor 1	−1.54	<0.05
C21-steroid hormone			
M21208mRNA_s_at	Cytochrome P450, subfamily 17	−2.83	<0.01
Bile acid metabolism			
S80431_s_at	Aldo-keto reductase family 1, member D1 (Δ4-3-ketosteroid-5-β-reductase)	−3.04	<0.05
U89905_at	α-Methylacyl-CoA racemase	−1.99	<0.01
D43964_at	Bile acid-CoA:amino acid *N*-acyltransferase	−1.57	<0.05

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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 cholesteryl 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.

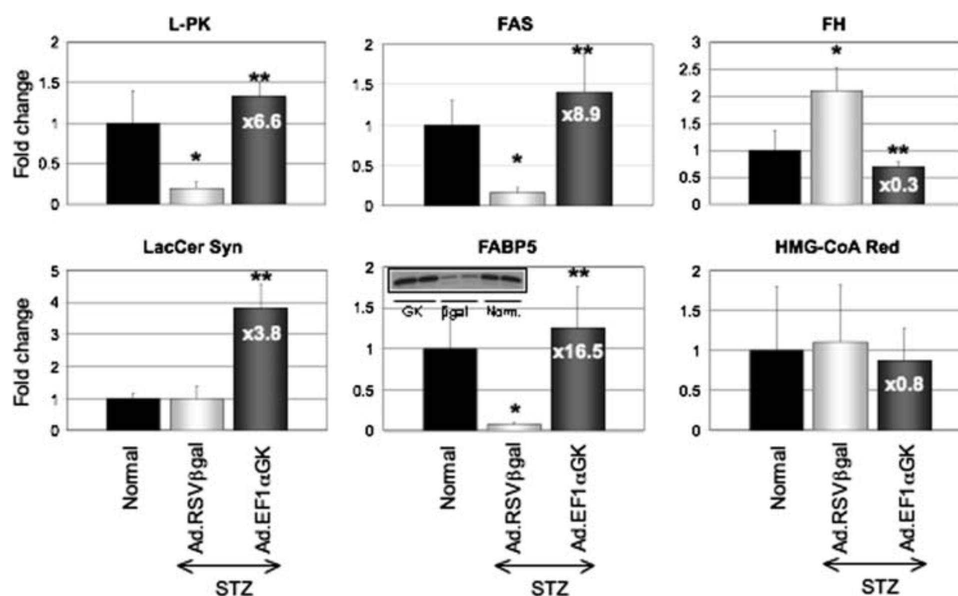

Fig. 1. RNA abundance based on real-time RT-PCR analysis. FABP5, fatty acid binding protein 5; FAS, fatty acid synthase; FH, fumarate hydratase; HMG-CoA Red, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LacCer Syn, lactosylceramide synthase; L-PK, liver-type pyruvate kinase; STZ, streptozotocin. 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.

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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

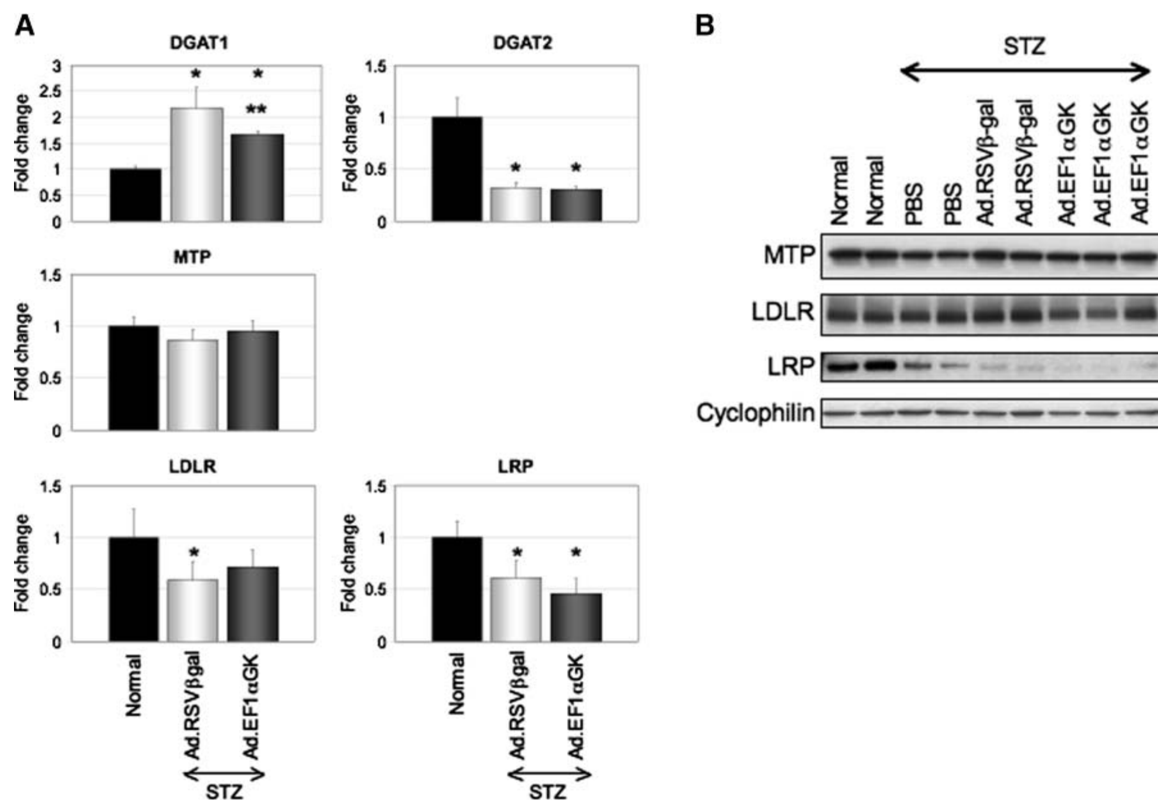

Fig. 2. RNA and protein abundance of genes involved in VLDL assembly and secretion. A: Real-time RT-PCR analysis. B: Western blot analysis. DGAT, diacylglycerol acyltransferase; LDLR, low density lipoprotein receptor; LRP, LDLR-related protein 1; MTP, microsomal transfer protein. Data shown are expressed as means ± SD. * $P < 0.05$ compared with the nondiabetic group; ** $P < 0.05$ between Ad.RSVβgal-treated rats and Ad.EF1αGK-treated rats.

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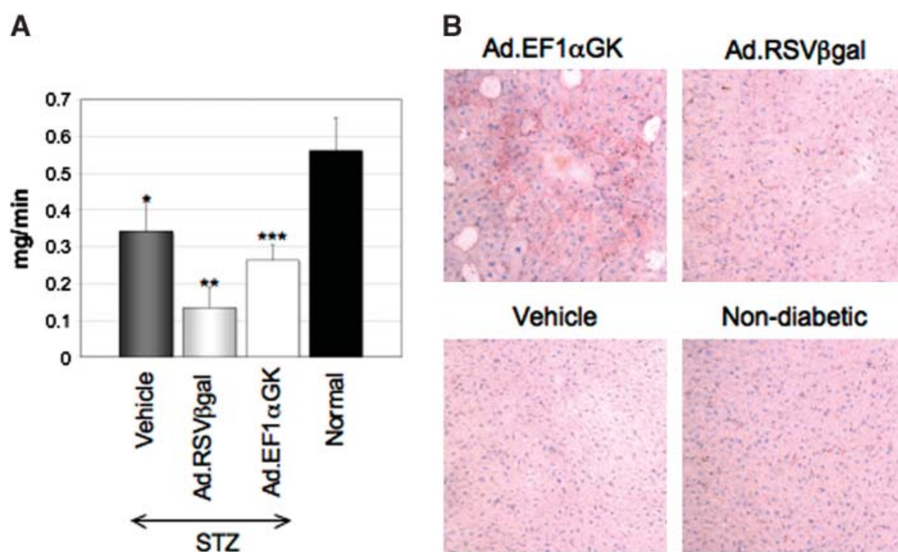

Fig. 3. Hepatic triglyceride secretion rate. A: On day 8 after virus administration, rats were fasted for 4.5 h and given a bolus of tyloxapol. The secretion rate was calculated from triglyceride levels in samples obtained at 0, 30, 60, and 90 min after tyloxapol administration, as described in Methods. Data shown are expressed as means ± SD. * Significantly different from the normal group ($P < 0.05$); ** significantly different from all other groups ($P < 0.05$); *** significantly different from the Ad.RSVβgal-treated and normal groups ($P < 0.05$). B: Oil Red O staining of liver sections of nondiabetic and vehicle-, Ad.EF1αGK-, and Ad.RSVβgal-treated diabetic rats euthanized on day 9.

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

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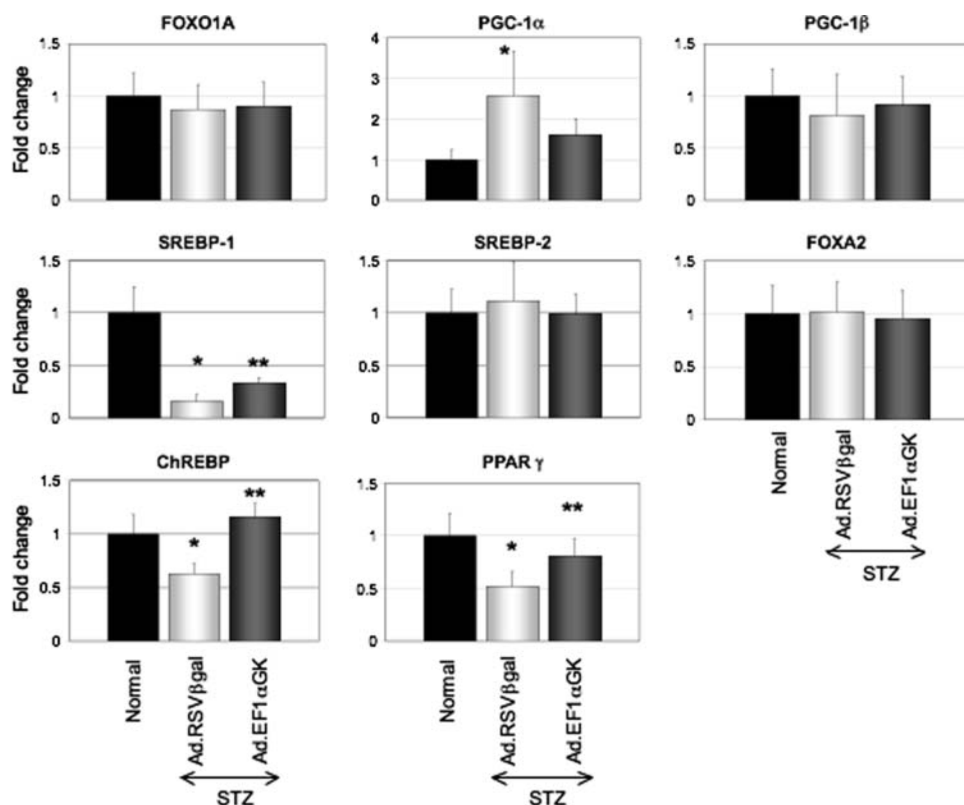

Fig. 4. RNA abundance of transcription factors/coactivators involved in glucose and fatty acid metabolism. ChREBP, carbohydrate-responsive element binding protein; FOXA2, forkhead box A2; FOXO1A, forkhead box O1A; PGC-1, PPARγ coactivator-1; PPARγ, peroxisome proliferator-activated receptor γ; SREBP, sterol-regulatory element binding protein. 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 animals.

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.EF1αGK 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.

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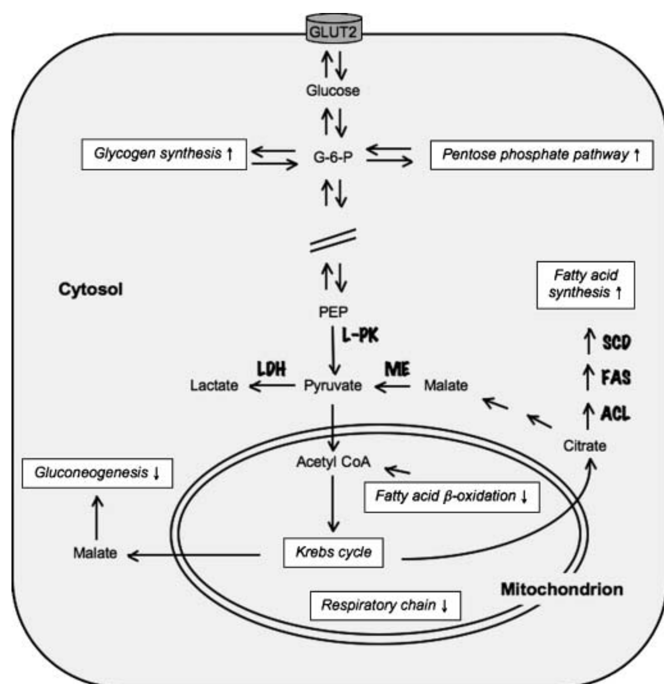

Fig. 5. Simplified scheme showing the major pathways upregulated or downregulated in the liver of glucokinase-overexpressing rats. ACL, ATP citrate lyase; FAS, fatty acid synthase; GLUT-2, glucose transporter 2; G-6-P, glucose-6-phosphate; LDH, lactate dehydrogenase; L-PK, liver-type pyruvate kinase; ME, malic enzyme; PEP, phosphoenolpyruvate; SCD, stearoyl-coenzyme A desaturase.

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 from DNL or from extracellular NEFAs (40–42). Recent data has suggested an additional biological role for DNL besides participating in triglyceride synthesis. Mice deficient for fatty acid synthase in liver are unable to activate PPARα and develop hepatic steatosis when fed a zero-fat diet (43). These effects are reversed by the administration of PPARα agonists or by feeding the animals a chow diet. These data suggest that "new" fatty acids (synthesized de novo or absorbed from the diet) are implicated in the activation of PPARα (43). Thus, it would make sense that DNL gene expression is not coupled to secretion, because the fate of the newly synthesized fatty acids may be short-term storage and/or translocation to the nucleus to activate PPARα.

Triglyceride levels in the circulation in glucokinase-overexpressing rats were significantly lower than those in rats treated with a control virus, suggesting a possible increase in lipoprotein uptake. This has been observed in mice overexpressing the transcription factor SREBP-1a, which controls the expression of genes of the fatty acid and cholesterol synthesis pathways, including the LDLR. SREBP-1a transgenic mice had markedly increased amounts of the lipogenic genes fatty acid synthase, acetyl-CoA carboxylase, and stearoyl-CoA desaturase as well as of LDLR and lipoprotein lipase (44). Increases in the levels of these genes resulted in low plasma triglyceride levels (44, 45). We did not observe significant differences in the levels of LDLR and LDLR-related protein 1 between glucokinase-overexpressing and control animals (Fig. 2). It is possible that another gene(s) involved in the uptake of lipoproteins is upregulated in the Ad.EF1αGK-treated rats or that, in these diabetic rats, the low circulating levels of glucose forced peripheral tissues to use fatty acids as an energy source, increasing the uptake of triglycerides. The low levels of circulating triglycerides in the Ad.EF1αGK-treated rats are in contrast with what has been reported in normal rats overexpressing glucokinase, which displayed plasma levels of triglycerides 190% higher than did control rats (46). Thus, insulin triggered the appropriate signals to induce the secretion of triglycerides into the circulation (46), whereas glucose metabolism (this study) did not induce such a response and fatty acids accumulated in the liver.

Until recently, the rate of DNL in humans (estimated by indirect calorimetry) was generally believed to be low (47). 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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