Insulin-Independent Induction of Sterol Regulatory Element–Binding Protein-1c Expression in the Livers of Streptozotocin-Treated Mice

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Insulin and glucose together have been previously shown to regulate hepatic sterol regulatory element–binding protein (SREBP)-1c expression. We sought to explore the nutritional regulation of lipogenesis through SREBP-1c induction in a setting where effects of sugars versus insulin could be distinguished. To do so, mice were insulin depleted by streptozotocin (STZ) administration and subjected to a fasting-refeeding protocol with glucose, fructose, or sucrose. Unexpectedly, the insulin-depleted mice exhibited a marked induction of SREBP-1c on all sugars, and this increase in SREBP-1c was even more dramatic than in the non-STZ–administered controls. The time course of changes in SREBP-1 induction varied depending on the type of sugars in both control and STZ-administered mice. Glucose refeeding gave a peak of SREBP-1c induction, whereas fructose refeeding caused slow and gradual increments, and sucrose refeeding fell between these two responses. Expression of various lipogenic enzymes were also gradually increased over time, irrespective of the types of sugars, with greater intensities in STZ-administered than in nontreated mice. In contrast, induction of hepatic glucokinase and suppression of phoshoenolpyruvate carboxykinase were insulin dependent in an early refed state. These data clearly demonstrate that nutritional regulation of SREBP-1c and lipogenic genes may be completely independent of insulin as long as sufficient carbohydrates are available.
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Sterol regulatory element–binding proteins (SREBPs) are members of the basic helix-loop-helix leucine zipper family of transcription factors that regulate fatty acid and cholesterol synthesis (1,2). SREBP proteins are initially bound to the rough endoplasmic reticulum membrane and form a complex with SREBP-cleavage activating protein, a sterol-sensing molecule (3,4). Upon sterol deprivation, SREBP is cleaved to liberate the amino terminal portion containing a basic helix-loop-helix leucine zipper domain (nuclear SREBP) and enters the nucleus, where it can bind to specific sterol response elements in the promoters of target genes (5). Three isoforms of SREBP, -1a, -1c, and -2 are known. Although SREBP-2 plays a crucial role in regulation of cholesterol synthesis, SREBP-1c controls gene expression of lipogenic enzymes (6–8).

Lipogenic enzymes, including genes such as acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD)-1 and -2, ATP citrate lyase, glucose-6-phosphate dehydrogenase, spot 14 (S14), and glycerol-3-phosphate acyltransferase, are a group of genes involved in energy storage through fatty acid and triglyceride synthesis (9,10). Excess amounts of carbohydrates taken up by cells are converted to triglycerides through these enzymes in liver and adipose tissue. The lipogenic enzymes are coordinately regulated at the transcriptional level by SREBP-1c during different metabolic states (11–15). Investigation on new SREBP targets led to identification of some new lipogenic enzyme genes such as acetyl CoA synthase and fatty acid elongase (16–19). Efforts to understand nutritional regulation of lipogenic enzymes focused on the promoter analysis of SREBP-1c gene expression as nuclear SREBP-1c activation is associated with SREBP-1c gene induction. Auto-loop activation through sterol response elements (20) and identification of liver X receptor–retinoid X receptor as an activator of the SREBP-1c promoter (21) have been reported; however, precise mechanisms of energy perception leading to activation of the SREBP-1c promoter have yet to be elucidated.

In liver, the presence of elevated concentrations of both glucose and insulin are required for the production of fatty acids via the induction of lipogenic enzymes (22,23). In

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ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; G6Pase, glucose-6-phosphatase; IRS, insulin receptor substrate; PEPCK, phoshoenolpyruvate carboxykinase; S14, spot 14; SCD, stearoyl-CoA desaturase; SREBP, sterol regulatory element–binding protein; STZ, streptozotocin; TBS-Ca, Tris-buffered saline containing 1 mmol/l $CaCl_2$.

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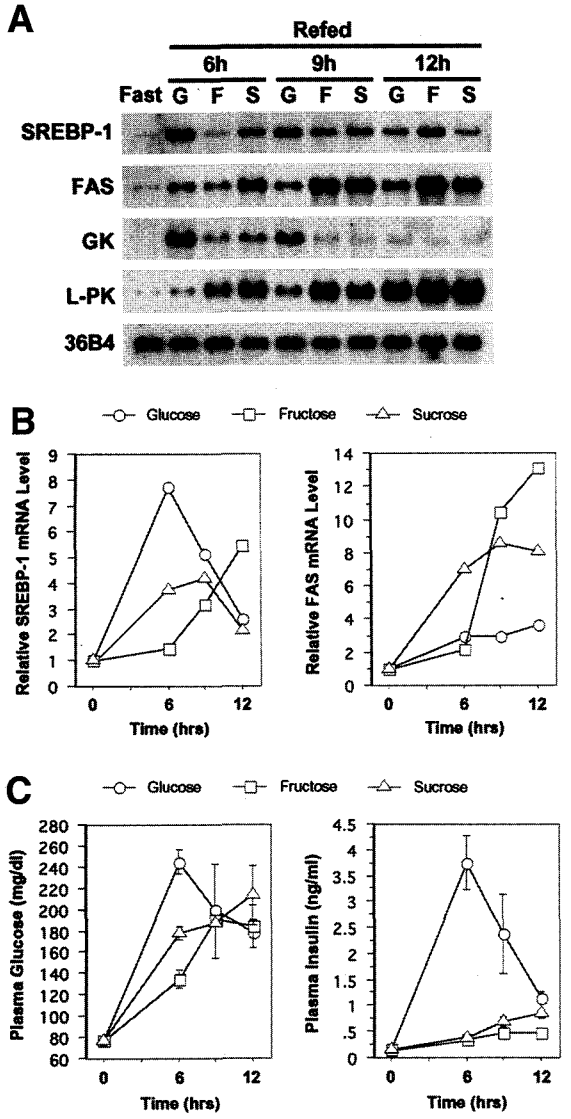

FIG. 1. Time-course changes of hepatic mRNA levels of SREBP-1 and lipogenic enzymes in the mice refed with glucose (G, ○), fructose (F, □), or sucrose (S, △). *A*: Northern blot analysis of different mRNAs in livers of mice. For each experimental group, total RNA isolated from the livers of three male mice was pooled, and 10-μg aliquots were subjected to electrophoresis and Northern blotting, followed by hybridization with the indicated ^{32}P-labeled cDNA probes. The blots are representative of three different experiments. *B*: Quantification of hepatic mRNAs for SREBP-1 and FAS. The data in panel *A* were quantified as described in RESEARCH DESIGN AND METHODS and normalized relative to the 36B4 (acidic ribosomal phosphoprotein p0) mRNA signal. The fold change is the relative ratio of each signal versus the corresponding fasted control mice, corrected for the signal from 36B4 as loading control. *C*: Plasma glucose and insulin levels of the mice fasted for 24 h and refed glucose, fructose, or sucrose for 6, 9, or 12 h. Results are the means ± SE of four male mice. GK, glucokinase; L-PK, L-pyruvate kinase.

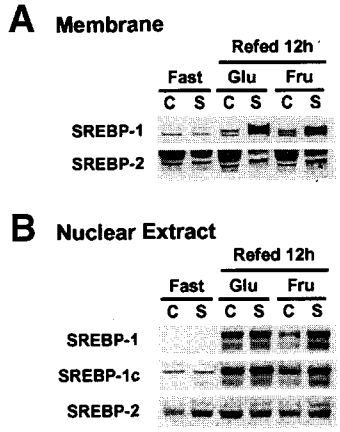

FIG. 2. Immunoblot analysis of SREBP-1 and -2 in membranes and nuclear extracts from the livers of control and STZ-administered mice fasted for 24 h and refed either glucose or fructose for 12 h. Livers from the six groups of mice (three male mice per group) were pooled, and aliquots of the membrane pellet (30 μg protein) (*A*) and nuclear extract (25 μg) (*B*) were subjected to SDS-PAGE. Immunoblot analysis was performed with rabbit anti-mouse SREBP-1 or anti-mouse SREBP-2 as the primary antibody and goat anti-rabbit horseradish peroxidase–conjugated IgG as the secondary antibody. Membranes were exposed to Eastman Kodak XAR-5 film. The blots are representative of two different experiments.

support of a role for SREBP-1c in the induction of lipogenic genes by insulin, several different groups (24–30), through various lines of evidence, have shown that SREBP-1c is upregulated by insulin in vivo and in primary hepatocyte cultures. Refeeding after fasting is well established as a strong stimulus for insulin secretion and is also the most potent stimulus known for induction of SREBP-1c and for the concomitant upregulation of lipogenic enzymes in vivo. However, it has also been shown that glucose is required for lipogenic enzymes where insulin only plays a permissive role (31–33). In addition, we and others (24,34) have shown that glucose can stimulate hepatic SREBP-1c expression. While it is clear that insulin facilitates this nutritional process, previous studies have shown that in several lines of insulin-resistant animals in which insulin signaling is somehow impaired, hepatic SREBP-1c expression is instead activated. These include leptin-deficient obese *ob/ob* mice, lipoatrophic mice overexpressing SREBP-1c in adipose tissue, insulin receptor substrate (IRS)-2 knockout mice, and rat primary hepatocytes infected with adenovirus-overexpressing, dominant-negative Akt (30,35,36). These data led us to speculate that SREBP-1c induction might not be a consequence of direct insulin signaling. To estimate the effects of sugars in insulin depleted states by streptozotocin (STZ) administration, the experiments were performed with a fasting-refeeding protocol in mice fed with different sugar diets. Our data demonstrate that SREBP-1c and lipogenesis can be induced fully in refed, insulin-depleted, STZ-administered mice, indicating that SREBP-1c induction does not require insulin.

RESEARCH DESIGN AND METHODS

Materials. Sugars were obtained from Wako Pure Chemical Industries. STZ was purchased from Sigma and insulin from Eli Lilly. Enhanced chemiluminescence Western blot detection kit and redivue [α-^{32}P]dCTP (6,000 Ci/mmol) were purchased from Amersham Pharmacia.

Animals and treatment. Seven-week-old male C57BL/6J mice (21–23 g) were purchased from Clea (Tokyo, Japan) and adapted to the environment for 1 week before initiation of the study. All mice were housed in a controlled environment with a 12-h light/dark cycle and free access to water and standard laboratory diet. Diabetes was induced by two intraperitoneal injections of STZ (100 mg/kg body wt; Sigma) with 1-day intervals. STZ was dissolved in 50 mmol/l sodium citrate buffer (pH 4.5) immediately before administration. Animals were considered diabetic when blood glucose levels exceeded 350 mg/dl, usually within 7 days from the first injection. For dietary studies, control and STZ-administered mice were fasted for 24 h and refed glucose, fructose, or sucrose for 3, 6, 9, or 12 h. STZ-administered mice injected with insulin received a combination of human regular insulin (3 units; Eli Lilly) subcutaneously and human NPH insulin (0.6 units; Eli Lilly)

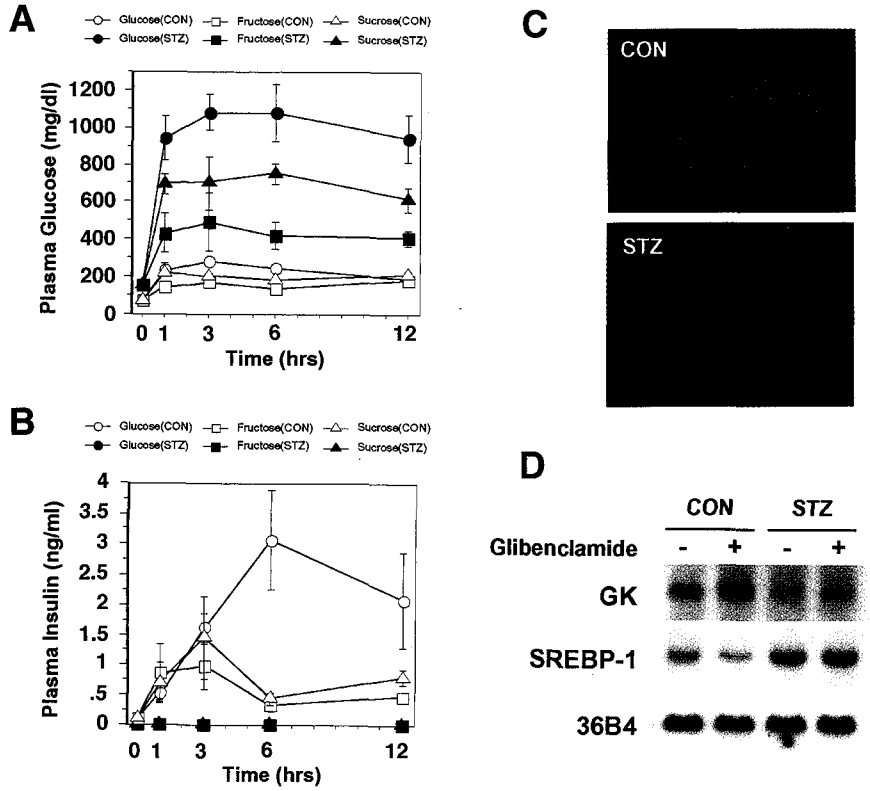

FIG. 3. Insulin depletion in STZ-administered mice. Time-course changes of plasma glucose (*A*) and insulin (*B*) levels from the control (CON) and STZ-administered (STZ) mice. Mice were fasted for 24 h and refed glucose, fructose, or sucrose, and blood samples were taken at the time indicated. Plasma glucose and insulin were determined as indicated in RESEARCH DESIGN AND METHODS. STZ-administered mice had no detectable levels of plasma insulin (<0.15 ng/ml) at any time point. Results are the means ± SE of five male mice. *C*: Formalin-fixed pancreas sections from control (CON) and STZ-administered (STZ) mice were stained for insulin as described in RESEARCH DESIGN AND METHODS. There was decreased insulin staining in the islets of STZ-administered mice. *D*: Sulfonylurea-induced insulin response in control (CON) and STZ-administered (STZ) mice. Glibenclamide (10 mg/kg) was administered intraperitoneally into control and STZ-administered mice that were refed with glucose for 1 h after fasting for 24 h. After an induction of hepatic glucokinase (GK), gene expression 6 h after glibenclamide treatment was examined by Northern blot analysis. For each experimental group, total RNA isolated from the livers of three male mice was pooled and 10-μg aliquots were subjected to electrophoresis and Northern blotting, followed by hybridization with the indicated ^{32}P-labeled cDNA probes. The blots are representative of two different experiments. Plasma insulin levels were 2.6 ± 0.4 and 3.1 ± 0.3 ng/ml for control and control injected with glibenclamide groups, respectively. STZ and STZ injected with glibenclamide groups had no detectable levels of plasma insulin (<0.15 ng/ml). Plasma glucose levels were 234 ± 31, 152 ± 15, 1,039 ± 60, and 841 ± 49 for the control, control injected with glibenclamide, STZ, and STZ injected with glibenclamide groups, respectively. Results are the means ± SE of three male mice.

intraperitoneally. The mice in a control group received PBS intraperitoneally and subcutaneously.

Male Wistar rats (250–300 g; Clea) were housed in a controlled environment with a 12-h light/dark cycle and free access to water and standard laboratory diet. After the animals were fasted for 24 h, rats in the STZ group were injected intravenously with 60 mg/kg body wt STZ in 50 mmol/l sodium citrate buffer (pH4.5), and rats in the control group were injected with 50 mmol/l sodium citrate solution. Tail vein blood glucose levels were measured, and animals were killed 1 week after the induction of diabetes. For dietary studies, control and STZ-administered rats were fasted for 48 h and refed either glucose, fructose, or sucrose for 24 h.

Blood chemistries and liver lipid analyses. The concentration of glucose, insulin, cholesterol, triglycerides, and nonesterified fatty acids in plasma and of cholesterol and triglycerides in liver were measured as previously described (37). Plasma leptin was determined by the mouse leptin enzyme-linked immunosorbent assay kit (Morinaga).

Immunoblot assays. Nuclear extracts and membrane fractions of mouse livers were prepared as previously described (38). A small portion of each

TABLE 1
Metabolic parameters in STZ-administered mice

			Refed 12 h					
	Fasted		Glucose		Fructose		Sucrose	
Parameter	Control	STZ	Control	STZ	Control	STZ	Control	STZ
Body weight (g)	20.0 ± 0.83	17.1 ± 0.51	21.5 ± 0.38	17.3 ± 0.34	21.1 ± 0.38	17.8 ± 0.31	20.8 ± 0.38	17.5 ± 0.34
Liver triglyceride (mg/g)	58 ± 8.7	3.9 ± 2.0	33 ± 8.5	5.9 ± 1.5	39 ± 4.1	7.6 ± 1.8	38 ± 8.9	7.3 ± 1.9
Liver cholesterol (mg/g)	1.9 ± 0.34	3.1 ± 0.24	2.4 ± 0.31	1.8 ± 0.17	2.2 ± 0.19	2.3 ± 0.18	2.0 ± 0.22	1.4 ± 0.94
Plasma triglyceride (mg/dl)	55 ± 8.0	62 ± 12.1	100 ± 9.5	30 ± 5.0	85 ± 10.5	36 ± 3.9	75 ± 9.3	43 ± 4.8
Plasma cholesterol (mg/dl)	107 ± 5.5	145 ± 10.6	82 ± 9.7	110 ± 8.1	103 ± 7.4	100 ± 9.0	83 ± 5.8	143 ± 12.4
Plasma NEFA (μmol/l)	1,297 ± 126	1,030 ± 253	298 ± 64	179 ± 33	410 ± 179	229 ± 47	146 ± 32	74 ± 7
Plasma leptin (ng/ml)	<0.2	<0.2	3.96 ± 0.23	<0.2	1.26 ± 0.17	<0.2	1.07 ± 0.10	<0.2

Data are the means ± SE of 5–8 male mice. Mice were fasted for 24 h and refed glucose, fructose, or sucrose for 12 h. NEFA, nonesterified fatty acid.

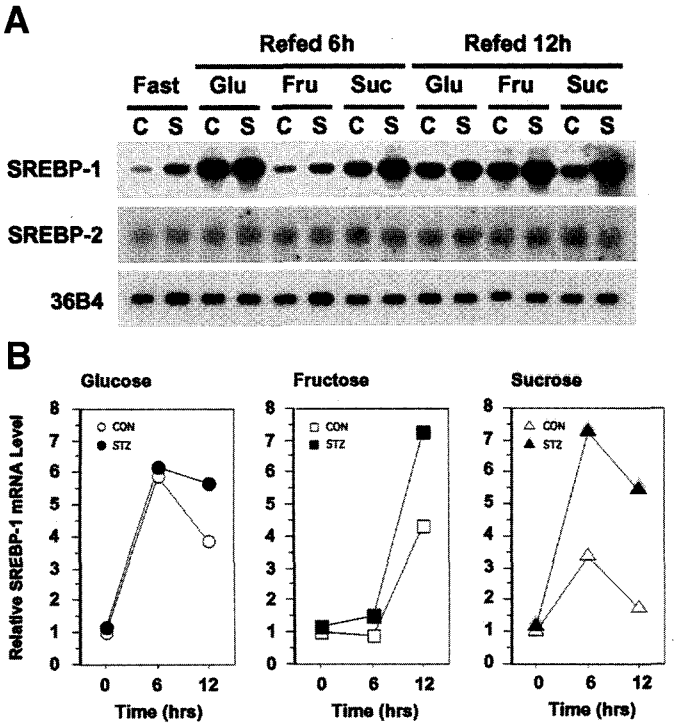

FIG. 4. Time-course changes of hepatic mRNA levels of SREBP-1 and -2 in the control and STZ-administered mice refed with glucose (Glu), fructose (Fru), or sucrose (Suc) for 6 and 12 h. *A*: Northern blot analysis of SREBP-1 and -2 mRNAs in the livers of control (C) and STZ-administered (S) mice. For each experimental group, total RNA isolated from the livers of three male mice were pooled, and 10-μg aliquots were subjected to electrophoresis and Northern blotting, followed by hybridization with the indicated ^{32}P-labeled cDNA probes. The blots are representative of three different experiments. *B*: Quantification of hepatic mRNA for SREBP-1. The data in panel *A* were quantified as described in RESEARCH DESIGN AND METHODS and normalized relative to the 36B4 (acidic ribosomal phosphoprotein p0) mRNA signal. The fold change is the relative ratio of each signal versus the fasted control mice, corrected for the signal from 36B4 as loading control.

sample was removed and analyzed for protein content by a bicinchoninic acid reaction (Pierce). Aliquots of nuclear (25 μg) and membrane (30 μg) proteins were subjected to immunoblot analysis with rabbit IgG against mouse SREBP-1 (11), SREBP-1c (15), and SREBP-2 (39), followed by horseradish peroxidase–linked goat IgG against rabbit IgG and the enhanced chemiluminescence kit (Amersham Pharmacia Biotech) (11,15).

RNA preparation and Northern blotting. Total RNA from liver was isolated with Trizol reagent (Invitrogen), and 10-μg RNA samples pooled equally from three mice of each group were run on a 1% agarose gel containing formaldehyde and transferred to Hybond-N membranes (Amersham Pharmacia Biotech). The cDNA probes for aldolase A, aldolase B, fructokinase, carbohydrate response element–binding protein, and GLUT2 were generated by using the sequence from GenBank with accession numbers Y00516, X53402, BC013464, AF156604, and BC034675, respectively. The other cDNA probes used were cloned as previously described (11,13,35). The probes were labeled with [α-^{32}P]dCTP using the Megaprime DNA labeling system kit (Amersham Biosciences). Membranes were hybridized with the radiolabeled probes in Rapid-hyb Buffer (Amersham Pharmacia Biotech) and washed in 0.1× SSC, 0.1% SDS, at 65°C. Blots were exposed to Eastman Kodak XAR-5 film, and the BAS imaging plate for the BAS2000 Bio Imaging Analyzer (Fuji Photo Film). The quantification results obtained with the BAS2000 system were normalized to the signal generated from 36B4 (acidic ribosomal phosphoprotein P0) mRNA.

Immunohistochemistry. Formalin-fixed pancreata were embedded in paraffin, and 4-μm–thick sections were used for insulin staining. After being dewaxed through xylene and absolute ethanol, the slides were rehydrated through decreasing concentrations of ethanol (100, 90, 80, and 70%) and rinsed in distilled water. The slides were washed thoroughly in Tris-buffered saline containing 1 mmol/l $CaCl_2$ (pH 7.6) (TBS-Ca) and incubated with 0.03% trypsin in TBS-Ca for 30 min. After blocking with TBS-Ca containing 5% skim milk for 1 h, the slides were exposed to the primary antibody, a polyclonal guinea pig anti-swine insulin (1:4; Dako) at 4°C overnight. After being washed in TBS-Ca, the slides were incubated with the secondary antibody, polyclonal sheep anti-guinea pig IgG conjugated to fluorescein isothiocyanate (1:100, PARIS) for 30 min at room temperature. After washing three times with TBS-Ca, the slides were photographed with fluorescence microscopy (Leica Microsystems).

RESULTS

To examine the kinetics of effects of different sugars on the expression of hepatic SREBP-1 and lipogenic enzymes, mice were fasted for 24 h and refed glucose, fructose, or sucrose for 6, 9, or 12 h, and the amount of mRNA was measured by Northern blotting (Fig. 1). Robust induction of SREBP-1 mRNA level was observed by all of the sugars but exhibited a considerable difference in their time-course patterns. When mice were refed with glucose, hepatic SREBP-1 gene expression was highest at 6 h and declined at 9 and 12 h postfeeding. In the livers of fructose-refed mice, the SREBP-1 mRNA level was at its peak at 12 h, exhibiting a delay in induction response compared with glucose. Livers refed with sucrose, which contains both glucose and fructose, showed a pattern between those with glucose and fructose. Plasma glucose and insulin levels were also compared. Glucose, the most potent insulin secretagogue, caused a higher response of both plasma glucose and insulin levels than fructose and sucrose refeeding, as expected. The overall induction patterns of SREBP-1 mRNA with each sugar did not necessarily follow the plasma insulin levels except in the case of glucose refeeding. Induction of FAS expression, a major target of SREBP-1, followed the changes in SREBP-1. Unexpectedly, glucose was a relatively weak activator of FAS mRNA compared with fructose and sucrose. In addition, the activation of hepatic glucokinase was highly insulin dependent.

Previous studies have focused primarily on insulin-induced expression of SREBP-1. To determine whether the carbohydrate regulation of the SREBP-1 was insulin dependent in vivo, STZ-administered mice were fasted for 24 h and refed either glucose or fructose for 12 h. Hepatic membrane-bound precursor forms and mature nuclear forms of SREBP-1 and -2 were compared and measured by immunoblot analysis (Fig. 2). When mice were fasted for 24 h, both membrane and nuclear forms of SREBP-1 were almost undetectable from either STZ-administered mice or nontreated controls. Refeeding with glucose or fructose for 12 h caused induction of SREBP-1 precursor protein in control mice, leading to incremental increases of nuclear proteins, a well-established observation in mice refed with a high-sucrose diet (13–15). Unexpectedly, in insulin-depleted mice, there was an even more profound increase in precursor SREBP-1 after glucose or fructose refeeding. Nuclear SREBP-1 protein was equally induced in STZ-treated mice. The induced isoform was SREBP-1c as depicted by a specific antibody, which is consistent with previous studies (14). There was no significant change in the precursor for SREBP-2. These results indicated that dietary glucose and fructose can highly induce active SREBP-1c protein independent of insulin.

Figure 3 shows time-course changes of plasma glucose and insulin levels in STZ-administered and nonadministered mice. All STZ-administered mice had undetectable levels of plasma insulin in any time point (1, 3, 6, and 12 h

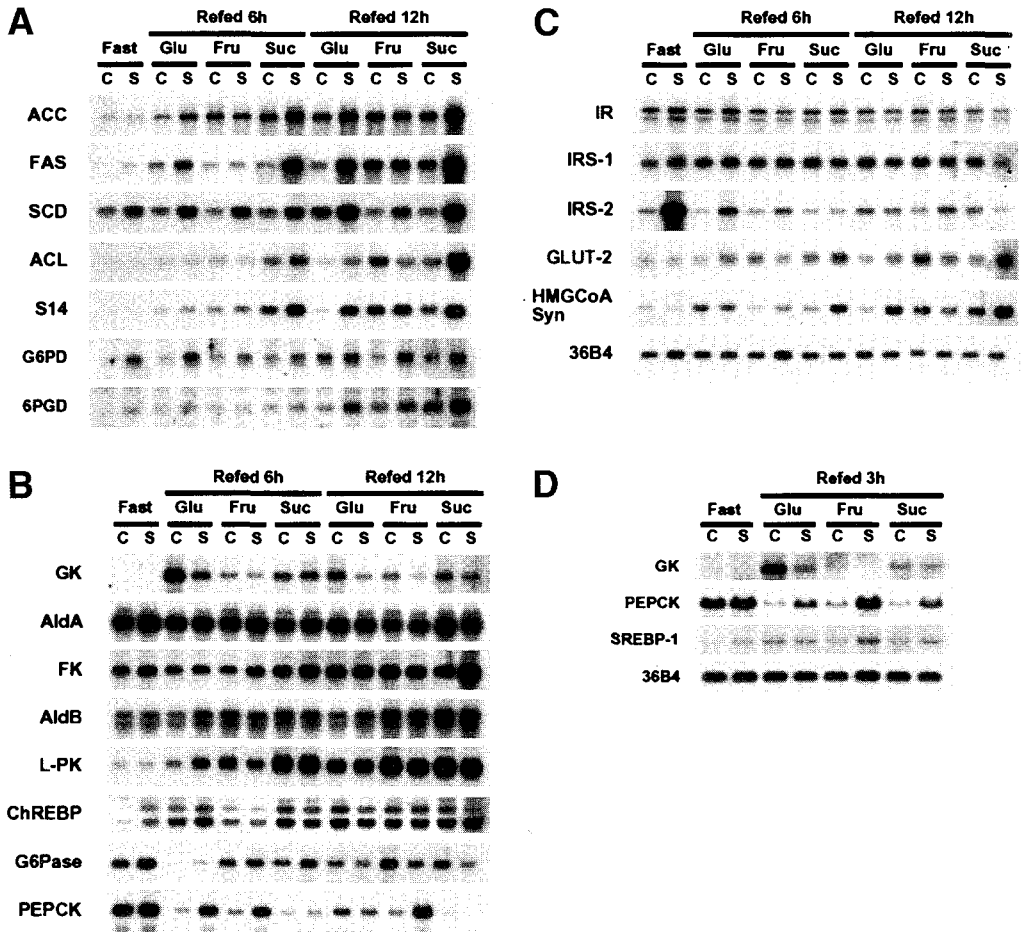

FIG. 5. Time-course changes of various mRNA levels in livers of the control and STZ-administered mice refed with glucose (Glu), fructose (Fru), or sucrose (Suc). *A*: Northern blot analysis of key genes in the regulation of lipogenesis in livers of control (C) and STZ-administered (S) mice refed with glucose (Glu), fructose (Fru), or sucrose (Suc) for 6 or 12 h. *B*: Northern blot analysis of key genes in the regulation of glycolysis and gluconeogenesis in livers of control (C) and STZ-administered (S) mice refed with glucose (Glu), fructose (Fru), or sucrose (Suc) for 6 or 12 h. *C*: Northern blot analysis of key genes in the regulation of insulin signaling, glucose transport, cholesterol synthesis, and 36B4 in livers of control (C) and STZ-administered (S) mice refed with glucose (Glu), fructose (Fru), or sucrose (Suc) for 6 or 12 h. *D*: Northern blot analysis of key genes in the regulation of glycolysis, gluconeogenesis, and SREBP-1 in livers of control (C) and STZ-administered (S) mice refed with glucose (Glu), fructose (Fru), or sucrose (Suc) for 3 h. For each experimental group, total RNA isolated from the livers of three male mice was pooled, and 10-μg aliquots were subjected to electrophoresis and Northern blotting, followed by hybridization with the indicated ^{32}P-labeled cDNA probes. The blots are representative of three different experiments. 36B4, acidic ribosomal phosphoprotein p0; 6PGD, 6-phosphogluconate dehydrogenase; ACL, ATP citrate lyase; Ald, aldolase; G6PD, glucose-6-phosphate dehydrogenase; GK, glucokinase; L-PK, pyruvate kinase; ChREBP, carbohydrate response element-binding protein; HMGCoA Syn, 3-hydroxy-3-methylglutaryl CoA synthase.

refeeding), confirming insulin depletion (Fig. 3*B*). Hyperglycemia was marked at a very early time point (1 h) and was sustained during the refeeding measured with glucose, sucrose, and fructose feeding, in this order of magnitude (Fig. 3*A*). Immunohistological examination of the pancreas showed that immunoreactive insulin in islets essentially disappeared after STZ administration, supporting complete depletion of plasma insulin (Fig. 3*C*). To assess a residual secretary function of β-cells in STZ-administered mice, we intraperitoneally administered glibenclamide to control and STZ-administered mice at the beginning of refeeding (1 h). The animals were further refed with glucose for 6 h for estimation of hepatic SREBP-1 and glucokinase mRNA levels (Fig. 3*D*). In control mice, glibenclamide treatment increased hepatic glucokinase expression as compared with nontreated mice. However, in STZ-administered mice, plasma insulin levels were still undetectable and hepatic glucokinase expression was not changed by glibenclamide treatment. These results strongly support that our STZ-administered mice had no residual secretory capacity of insulin and completely lost the effects of insulin.

Table 1 represents other parametric measurements of mice with STZ administration and of controls without treatment. STZ administration caused a significant decrease in body weight, with disappearance of fat, and a marked increase in plasma glucose. Plasma leptin levels were also very low, reflecting loss of adipose tissue. STZ administration also reduced plasma and hepatic triglycerides.

To determine the scope of insulin effects on gene expression patterns of carbohydrate/lipid metabolism, Northern blot analysis was performed on a variety of related genes in the livers of STZ-administered mice refed with each sugar at 6 and 12 h (Figs. 4 and 5). In concert with protein levels, the amount of hepatic SREBP-1 mRNA in STZ-administered mice was equally or even more highly induced by any sugar refeeding than in nontreated controls. The time course of changes in SREBP-1 mRNA levels by each sugar refeeding was similar between STZ-admin-

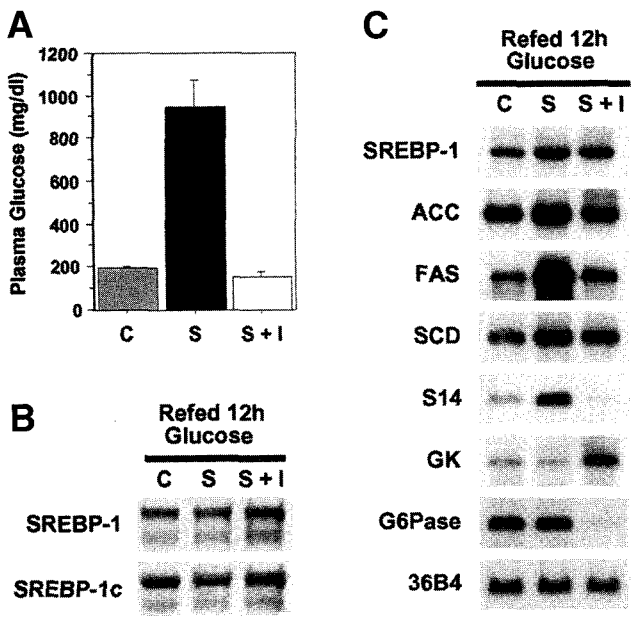

FIG. 6. Effect of insulin on the protein and mRNA levels in livers of control (C), STZ-administered (S), and STZ-administered mice injected with insulin (S + I) refed with glucose. *A*: Plasma glucose levels from the control (C), STZ-administered (S), and STZ-administered mice injected with insulin (S + I). Results are the mean ± SE of four male mice. *B*: Immunoblot analysis of SREBP-1 and -1c in nuclear extracts from pooled livers of each group. The primary antibodies used were polyclonal anti-mouse SREBP-1 and anti-mouse SREBP-1c. The blots are representative of two different experiments. *C*: Northern blot analysis of different mRNAs in livers of each experimental group. Total RNA isolated from the livers of four male mice was pooled, and 10-μg aliquots were subjected to electrophoresis and Northern blotting, followed by hybridization with the indicated ^{32}P-labeled cDNA probes. The blots are representative of three different experiments. 36B4, *acidic ribosomal phosphoprotein p0; GK, glucokinase.*

istered and control mice (Fig. 4). In contrast, there was no significant change in expression of SREBP-2 by fasting/refeeding or by STZ administration. As shown in Fig. 5*A*, expression of SREBP-1–sensitive lipogenic genes, such as ACC, FAS, SCD, ATP citrate lyase, S14, glucose-6-phosphate dehydrogenase, and 6-phosphoglucanate dehydrogenase, were steadily induced in a time-dependent manner by refeeding with any sugar, with the highest levels by sucrose seen at 12 h. Overall, STZ administration enhanced the induction of these genes. As for genes involved in glucose/fructose metabolism, the mRNA encoding glucokinase was reduced by STZ administration, especially in a fed glucose state (Fig. 5*B*). In contrast, the gluconeogenic enzyme mRNAs for PEPCK and glucose 6 phosphatase (G6Pase) were slightly increased by STZ administration at 6 and 12 h refeeding. In the glycolytic pathway, aldolase A did not show significant changes. Fructokinase, aldolase B, and liver-type pyruvate kinase, all of which are involved in lipogenesis from fructose, were not affected by STZ but showed a tendency of induction in a late stage of refeeding. The mRNA for IRS-2 increased slightly during fasting and dramatically after STZ administration, whereas there was no significant change in the mRNAs for the insulin receptor and IRS-1 (Fig. 5*C*). GLUT2 expression was increased in refed STZ-administered mice, assuring increased glucose uptake by hepatocytes in hyperglycemia. The mRNA for the cholesterologenic enzyme, 3-hydroxy-3-methylglutaryl CoA synthase, was also increased by STZ administration. 36B4 was used as a loading control.

To more precisely confirm insulin deficiency, we measured mRNA levels of glucokinase, PEPCK, SREBP-1, and IRS-2 at 3-h refeeding with glucose. At this early time point, SREBP-1 was not yet induced (Fig. 5*D*). Hepatic glucokinase gene expression in control mice was already highly induced but was almost completely suppressed by STZ administration. Conversely, high expression of PEPCK was already suppressed at 1-h refeeding, and this suppression was blunted in STZ-administered mice. Thus, at this time point, the expression of genes involved in glucose metabolism was under the control of insulin, as expected.

To determine whether any changes in SREBP-1 and lipogenic gene expression could be reversed, the STZ-administered mice were supplemented with insulin during glucose refeeding. As shown in Fig. 6, exogenous insulin completely normalized the plasma glucose levels of STZ-administered mice, but had no effect on the SREBP-1 mRNA level. Mature forms of SREBP-1c did not show significant changes by STZ administration or subsequent insulin supplementation, as estimated by immunoblot analysis. The mRNA levels of lipogenic enzymes, including ACC, FAS, SCD, and S14, were significantly elevated by STZ administration and, in mice given insulin, declined to the levels of non-STZ–administered controls. The predicted effects of insulin on hepatic gene expression were observed in induction of glucokinase and suppression of G6Pase. Plasma leptin levels were suppressed by STZ administration and partially restored by insulin supplementation (3.42 ± 0.40, 0.085 ± 0.026, and 1.92 ± 1.12 ng/ml for control, STZ, and STZ + insulin, respectively).

Since insulin dependence of SREBP-1 expression has been proposed from rat primary hepatocyte or STZ-administered rats (26), it is possible that the discrepancy in our data from mice could be due to a difference between species. To examine whether the regulation of the SREBP-1 gene expression in our protocol was insulin dependent in rats, we also performed Northern blot analysis in the livers of control and STZ-administered rats fasted for 48 h and refed either glucose, fructose, or sucrose for 24 h. As shown in Fig. 7, SREBP-1 expression was significantly decreased in the livers of STZ-administered rats, suggesting more insulin dependency in rat SREBP-1 expression. Expression of the lipogenic genes FAS and S14 was also repressed in the livers of STZ-administered rats. Thus, there is a considerable species difference in the dependency of hepatic SREBP-1 on insulin.

DISCUSSION

Insulin independence of SREBP-1c induction in a refed state. While previous reports have shown that insulin appears to induce SREBP-1 expression, our current data suggest that dietary activation of SREBP-1c in a refed state could be insulin independent in mouse livers at least. In fully refed states (12-h refeeding after 24 h fasting), SREBP-1c and its target lipogenic genes were highly induced by any sugar tested in insulin-depleted conditions by STZ administration. The similar induction of SREBP-1c and FAS expression was also observed in the STZ-administered mice refed with a regular diet (data not shown). Fructose can be efficiently taken up by hepatocytes with-

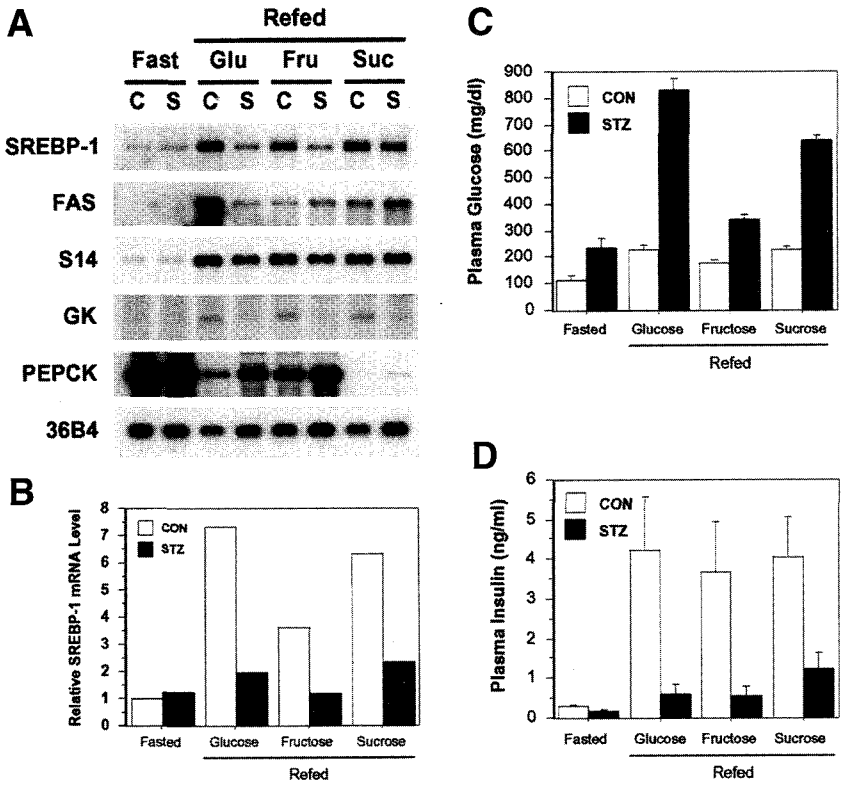

FIG. 7. A, Northern blot analysis of SREBP-1 and different mRNAs in livers of control (C) and STZ-administered (S) rats. For each experimental group, total RNA isolated from the livers of three male rats was pooled, and 10-μg aliquots were subjected to electrophoresis and Northern blotting, followed by hybridization with the indicated ^{32}P-labeled cDNA probes. The blots are representative of three different experiments. *B*: Quantification of hepatic mRNA for SREBP-1. The data in panel *A* were quantified as described in RESEARCH DESIGN AND METHODS and normalized relative to the 36B4 (acidic ribosomal phosphoprotein p0) mRNA signal. The fold change is the relative ratio of each signal versus the fasted control rats, corrected for the signal from 36B4 as loading control. Plasma glucose (*C*) and insulin (*D*) levels from the rats fasted for 48 h and refed glucose, fructose, or sucrose for 24 h are shown. Results are the means ± SE of three male rats.

out insulin (40). Therefore, fructose activation of SREBP-1c in normal mice is also supportive evidence for the insulin independence of SREBP-1c induction. Previous studies (29) suggesting that insulin regulates SREBP-1c expression were performed at short fasting times, when insulin dominates the metabolic fate of glucose. Lipogenesis is activated after glycogen synthesis is fully stored and excess energy is ready for lipid deposition. Insulin would be expected to facilitate this process. However, when expression of lipogenic genes was fully activated in glucose refeeding, plasma insulin levels were already decreasing (Fig. 1). Furthermore, previous studies suggest that chronic insulin exposure to hepatocytes causes secondary insulin resistance through IRS-2 repression, which is observed in a refed state (35,41). Considering this time lag in the process of these nutritional events, it is unlikely that the same insulin signal that mediates acute glucose metabolism, such as glycogen synthesis, also regulates SREBP-1c and lipogenic gene expression in a late phase of nutritional process. The insulin dependency of SREBP-1c expression in previous reports was all based on rats or rat primary hepatocytes. It is also possible that the discrepancy could be a species difference between rats and mice. We also estimated the hepatic SREBP-1c expression of refed STZ-administered rats. STZ administration significantly decreased SREBP-1c expression, suggesting more insulin dependency in rat SREBP-1c expression (Fig. 7).

Glucose dependency of SREBP-1c induction in a refed state. We have shown here that SREBP-1c induction was correlated with plasma glucose levels rather than plasma insulin levels in both STZ-administered and non-administered mice. Uptake of glucose by hepatocytes is mediated through GLUT2, which does not require insulin for activation (42), and was interestingly increased by STZ. Thus, cellular glucose availability is correlated with plasma glucose level. We and others (24,34) have postulated that glucose could induce hepatic SREBP-1c expression with the permissive action of insulin. The current data are consistent with this notion and, at least in a late fed phase of mice, insulin is not required for induction of hepatic SREBP-1 and lipogenic enzymes.

Metabolic pathways of fructose to lipogenesis in a refed state. SREBP-1c has been suggested to be involved in metabolic derangements in the liver as a part of the pathogenesis of the metabolic syndrome (43). Beside insulin and glucose, a diet rich in fructose has been known to induce hyperinsulinemia, hyperlipidemia, and fatty liver (44–46). Furthermore, a high-fructose diet was reported to induce hepatic SREBP-1c mRNA (47). After uptake by hepatocytes through GLUT2, fructose is converted to fructose-1-phosphate by fructokinase and thereafter enters into the glycolytic pathway as triose phosphates catalyzed by aldolase B, bypassing an insulin-regulated step by phosphofructokinase-1. Fructokinase and aldolase B are not direct SREBP targets because they are not induced in the livers of SREBP transgenic mice (data not shown). However, both genes showed time-dependent induction at refeeding irrespective of STZ administration,

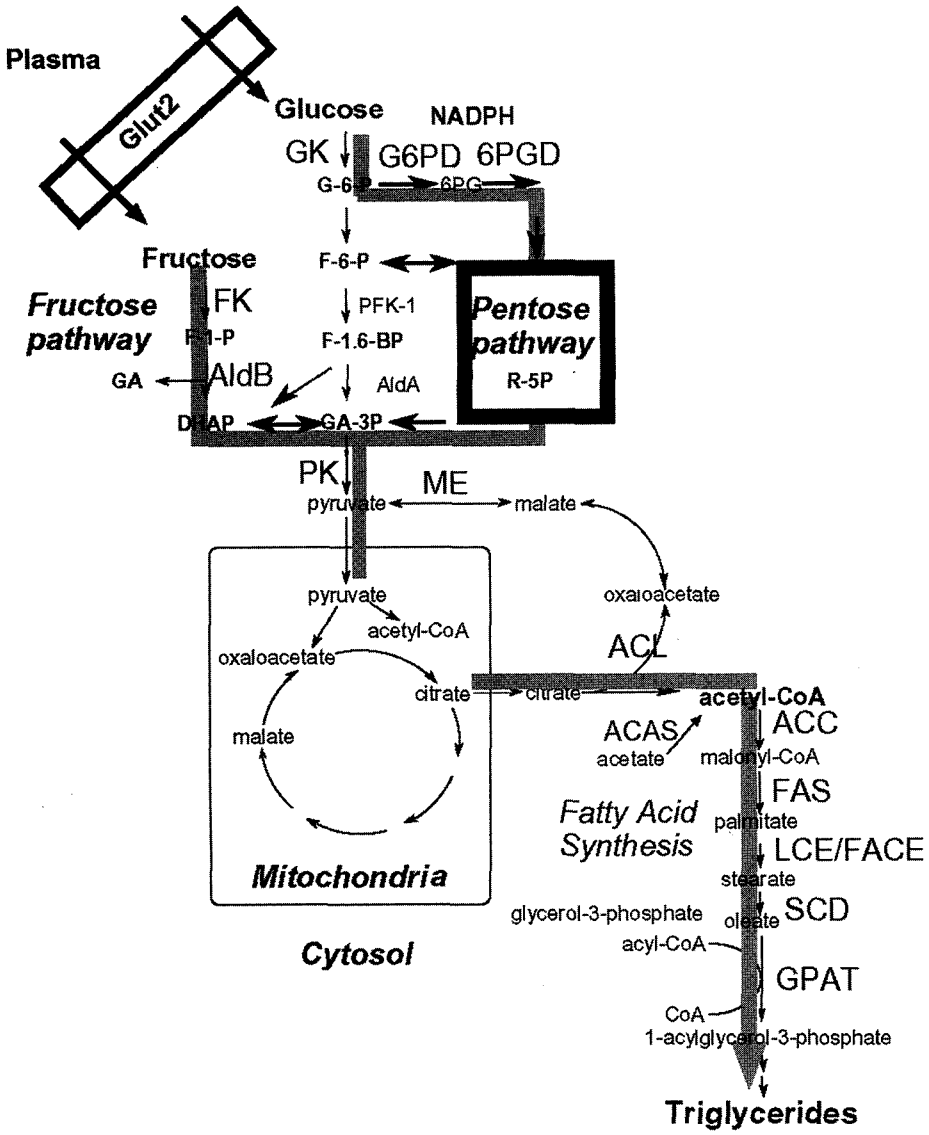

FIG. 8. Metabolic pathways for hepatic lipogenesis in mice refed with glucose and/or fructose. Major pathways for lipogenesis after ingestion of glucose or fructose are highlighted by bold lines. 6PG, 6-phosphogluconate; 6PGD, 6-phosphogluconate dehydrogenase; ACAS, acetyl-CoA synthase; ACL, ATP citrate lyase; Ald, aldolase; DHA-3P, dihydroxyacetone-3-phosphate; F-1,6-BP, fructose-1,6-bisphosphate; F-1-P, fructose-1-phosphate; F-6-P, fructose-6-phosphate; FACE, fatty acyl-CoA synthase; FK, fructokinase; G-6-P, glucose-6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; GA, glycelaldehyde; GA-3P, glyceraldehyde-3-phosphate; GK, glucokinase; Glut2, glucose transporter 2; GPAT, glycerol-3-phosphate acyltransferase; LCE, long-chain elongase; ME, malic enzyme; PFK-1, phosphofructokinase-1; PK, pyruvate kinase; R-5P, ribrose-5-phosphate.

which is evidence for the activity of this pathway in the refed condition. It is conceivable that in a refed state with fructose as the only sugar source, the triose phosphates could be used for both lipogenesis and glucose production to maintain blood glucose.

The activation of hepatic SREBP-1c and lipogenic genes by refeeding with pure glucose or fructose feeding illustrates two major metabolic pathways from these monosaccharides to lipogenesis, as is shown in Fig. 8. Both SREBP-1–activated pathways could bypass the phosphofructokinase-1–catalyzed step, which is a rate-limiting and insulin-regulated step under feedback regulation. The subsequent rate-limiting step, pyruvate kinase, although not an SREBP-1 target, was also highly induced in a refed state without insulin, presumably supported by concomitant induction of carbohydrate response element-binding protein, which was recently identified as a transcription factor for L-pyruvate kinase (48). These data indicate that glucose and fructose ingestion can very efficiently lead to the induction of lipogenic genes via these pathways without insulin.

SREBP-1–independent regulation of lipogenic enzymes in a refed state. Although the roles of SREBP-1c in the control of lipogenic enzymes have been well established, the current studies also support the presence of SREBP-1–independent control of lipogenic enzymes. STZ administration did not change nuclear SREBP-1 protein but still increased lipogenic enzymes such as FAS, ACC, SCD, and S14 (Fig. 6). Insulin supplementation in turn decreased expression of these enzymes to the levels of controls. These data suggest that changes in lipogenic enzymes related to insulin action may be caused by some factor other than SREBP-1.

Signaling molecule for induction of SREBP-1c and lipogenic genes. Because fructose feeding gradually induced SREBP-1c expression, it could be postulated that glucose and fructose need to be metabolized by some common substance for activation of SREBP-1c in a cumulative fashion. Insulin could enhance this conversion from glucose, which explains why refeeding normal mice with glucose causes the most rapid and potent activation of SREBP-1c, with a corresponding insulin increment (Fig. 1). But even in insulin depletion, severe hyperglycemia in STZ-administered mice presumably compensates for the lack of this effect of insulin. The identity of the parameter or signaling molecule for SREBP-1c induction is currently

an enigma. It could be some metabolite in the final common pathway, or this metabolic parameter could be related to an energy-sensing molecular ratio, such as ATP-to-AMP. Further investigations are needed for comprehensive elucidation of the regulation of the carbohydrate/lipid metabolism. It is also of great clinical importance as this energy-sensing mechanism is likely to be related to obesity and insulin resistance.

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