Restoration of direct pathway glycogen synthesis flux in the STZ-diabetes rat model by insulin administration

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Hepatic glycogen is synthesized via two pathways, the classical direct pathway from intact glucose units and the indirect pathway by which glucose is first metabolized to 3-carbon intermediates (pyruvate and further to lactate), followed by gluconeogenic resynthesis into glucose 6-phosphate (G6P) (Fig. 1) (43). This latter route also allows for nonhexose precursors such as glycerol and gluconeogenic amino acids to contribute to glycogen synthesis in the liver. It is well established that the direct pathway is the major route of hepatic glycogen synthesis (4, 5, 21, 22, 25). Hepatic glycogen synthesis fluxes in type 1 diabetes patients have been shown to respond to insulin therapy. Poorly controlled type 1 diabetes patients have reduced glycogen stores in the liver that can be increased, but not fully recuperated, with short-term intensive insulin therapy (5). On the other hand, after long-term intensive insulin therapy, net hepatic glycogen synthesis rates and peak postprandial glycogen levels were restored to those of healthy controls (4). However, the indirect pathway still accounted for the majority of glycogen synthesis, whereas direct pathway flux remained significantly depressed (4). To our knowledge, restoration of direct pathway fluxes in type 1 diabetes subjects has not been reported for any intervention. Therefore, it remains unclear whether the loss of direct pathway flux is an irreversible consequence of type 1 diabetes or whether it can be restored by insulin or other therapies.

Small-animal models such as the streptozotocin (STZ)-administered rat are important for understanding the pathophysiology of type 1 diabetes and for development of therapies to reverse its metabolic defects. Namely, the STZ rat model is characterized by persistent hyperglycemia, reduced hepatic glycogen content, and increased lipids in the liver (12, 14, 46). The metabolic fluxes that lead to high blood glucose levels, hepatic glycogen depletion, and lipid accumulation can be estimated in the experimental setting by using deuterated water (2H2O) as a tracer, followed by the determination of the 2H enrichment in glucose, glycogen, and triglycerides. Upon administration, 2H2O is distributed freely within the tissues and is rapidly equilibrated with total body water. For example, in rats, an intraperitoneal bolus of 2H2O results in a steady-state 2H enrichment of the body water within 10 min (60).

Recently, we reported a 2H2O-based method that allows hepatic glycogen synthesis via direct and indirect pathways to be determined in naturally feeding rodents (57). Our measurements revealed glycogen synthesis flux profiles in healthy and STZ rats that resembled those of healthy and type 1 diabetes subjects (57). Presently, we hypothesized that, as for type 1 diabetes subjects, defective hepatic glycogen fluxes in STZ rats could be corrected by insulin administration. Hence, we compared the effects of subcutaneous and intraperitoneal insulin (I-SC and I-IP, respectively) administration on postprandial hepatic glycogen fluxes in STZ rats. We further hypothesized that I-IP would be more effective at recapitulating the portal-peripheral insulin gradient and, therefore, be more effective at restoring hepatic glycogen fluxes.

Finally, in the same setup, the activity of the lipogenic pathway was assessed by measuring the 2H enrichment in the
hepatic triglycerides from the $^2$H$_2$O tracer (10, 40, 55). It has been shown previously that, in the STZ rat, there is a down-regulation of de novo lipogenesis (DNL) (38) and that the lipogenic flux from $^{13}$C-enriched precursors is strongly impaired in perfused livers from STZ rats (9). In this study, we were able to measure the DNL pathway in whole animals and investigate the effect of diabetes and I-SC and I-IP on that pathway under ad libitum conditions.

Our data demonstrate that glycogenic and lipogenic pathways were responsive to insulin administration in these models despite an inefficient glycemic control throughout the day course. Notably, and in sharp contrast to human type 1 diabetes subjects, direct pathway fluxes of glycogen synthesis were completely restored by I-SC as well as I-IP.

METHODS

Ethics Statement

Animal study protocols conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee at the Center for Neuroscience and Cell Biology of the University of Coimbra.

Animal Studies

Male Wistar rats weighing 280 ± 2 g with free access to standard chow over a 12:12-h light-dark cycle were given a 65 mg/kg intraperitoneal injection of STZ. On day 9 post-STZ, nonfasted animals with glycemia >250 mg/dl were randomly assigned into three groups. Two groups of animals received two daily injections of short-acting insulin (Actrapid; Novo Nordisk) at a dose of 15 U/kg subcutaneously (I-SC; $n = 9$) or intraperitoneally (I-IP; $n = 9$). Injections were given 1 h into the fight cycle and at the start of the dark cycle. This twice-daily injection protocol of short-acting insulin allowed us to recreate a model of poorly controlled type 1 diabetes. The third group was left untreated (STZ; $n = 7$). Blood glucose and triglyceride levels were monitored with Accu-Chek and Accutrend (Roche, Amadora, Portugal) meters, respectively. On day 18 post-STZ, animals received an intraperitoneal injection of saline in 99% $^2$H$_2$O (2 g/100 g body water) 30 min after the dark cycle insulin injection, and their drinking water was enriched to 2% with $^3$H$_2$O (57). Then the animals were left to feed ad libitum over the dark cycle (~12 h) and were euthanized the next morning by ketamine (100 mg/kg) intraperitoneal injection. The $^2$H$_2$O protocol was also applied to a separate group of healthy weight-matched control animals (non diabetic controls; $n = 5$). After euthanization, blood for metabolite analyses was collected from the descending aorta; the liver was excised and freeze-clamped immediately in liquid nitrogen, and epididymal white adipose tissue (EWAT) was collected and weighed.

Metabolite Analyses

Plasma levels of endogenous insulin and exogenous insulin were determined by enzyme-linked immunooassay (Mercodia, Upsala, Sweden), using specific assay kits for rat and human insulin, respectively; plasma nonesterified fatty acids (NEFA) were determined with a NEFA-HR assay kit (Wako Chemicals, Neuss, Germany). Plasma glucose was derivatized to monoacetone glucose (MAG), as described previously (56). Liver was lyophilized and pulverized to a fine powder. Two-thirds was treated with 30% KOH followed by ethanol to recover glycogen, which was then hydrolyzed to glucose with amyloglucosidase (Sigma-Aldrich, Sintra, Portugal) and converted to MAG, as described previously (56, 57). An aliquot was saved for quantification with Invitrogen Amplex Red glucose assay kit (Alfagene, Carcavelos, Portugal). Concentrations were expressed as micromoles per gram dry weight of liver. Dry weight values were determined by direct weighing of lyophilized livers. The remaining one-third was extracted for lipids by the Folch method.

NMR Spectroscopy

Body water $^2$H$_2$O enrichments were determined from plasma by $^2$H-NMR, as described previously (23). $^2$H-NMR spectra of MAG were obtained at 50°C with a 14.1 T Varian 600 system (Agilent, Palo Alto, CA) equipped with a 3-mm broadband probe. Fully relaxed spectra were acquired with 4,000–25,000 scans, a 90° pulse, an acquisition time of 1.6 s, and a pulse delay of 0.1 s. Field frequency stability was maintained via the Varian 1H-Scout acquisition sequence. $^1$H- and $^2$H-NMR spectra of lipid extracts were acquired at 25°C in the same spectrometer with a 5-mm broadband probe. $^1$H-NMR spectra of lipids consisted of a single scan using a 45° pulse and an acquisition time of 3 s, whereas $^2$H-NMR spectra of lipids were acquired with 200–8,000 scans using a 90° pulse, an acquisition time of 5 s, and a pulse delay of 2 s. Body water, MAG, and triglyceride $^2$H enrichments were quantified from $^2$H-NMR spectra, as described previously (10, 23). Spectra were analyzed with the NUTS program (Acorn NMR, Aliso, CA) equipped with a 3-mm broadband probe. Fully relaxed spectra were acquired with 4,000–25,000 scans, a 90° pulse, an acquisition time of 1.6 s, and a pulse delay of 0.1 s. Field frequency stability was maintained via the Varian 1H-Scout acquisition sequence.

Quantitative RT-PCR Analysis

Total RNA was isolated from frozen liver using the Speedtools Total RNA Extraction Kit (Biotools, Madrid, Spain). Single-stranded complementary DNA templates for PCR amplification were synthesized from 1 μg of total RNA by incubation with reverse transcriptase (M-MLV RT; Promega Biotech Iberia, Madrid, Spain) at 37°C for 1 h. The cDNA was diluted ×20 in milliQ water, and real-time quantitative PCR was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA) using 0.4 μmol/l of each primer, 10 μl of SYBR Green (Applied Biosystems), and 1.6 μl of diluted cDNA. The temperature cycle protocol for amplification was 95°C for 10 min, followed by 40 cycles with 95°C for 15 s and 62°C for 1 min. Variations in gene expression were calculated by the standard ΔΔCT method using ribosomal subunit 18 as endogenous control.

Enzyme Activities

Enzyme activity assays and total protein content were adapted for automated measurement using a Cobas Mira S spectrophotometric analyzer (Hoffman-La Roche, Basel, Switzerland), as described previously (7, 36). Total protein content was determined by the Bradford method (Bio-Rad Laboratories, Barcelona, Spain) at room tempera-
tured at 600 nm using bovine serum albumin as a standard. Enzyme assays were adapted for 4–5 μl of liver extract, performed at 30°C, and followed at 340 nm.

**Hepatic Metabolic Fluxes**

_Glycogen repletion during overnight feeding._ After the $^2$H$_2$O loading dose at the start of the dark period, a constant level of body water $^2$H enrichment was rapidly achieved and maintained throughout the night. Preexisting (i.e., preprandial) glycogen remained unlabeled with $^3$H, whereas any glycogen synthesized after $^3$H$_2$O administration was enriched in position 2 to the same level as body water (BW) due to extensive exchange of G6P with fructose 6-phosphate (22, 26). Therefore, any reduction or dilution of glycogen position 2 enrichment relative to that of BW is attributed to preprandial glycogen. On this basis, the fraction of newly synthesized glycogen was calculated as follows:

$$\text{Newly synthesized fraction (\%)} = 100 \times \frac{H2/BW}{H5/H2}$$

where $H2$ is the $^2$H enrichment of position 2 and $BW$ is the $^2$H enrichment of body water.

Preprandial hepatic glycogen levels at the start of the dark cycle were calculated as the difference between total glycogen levels measured at the end of the experiment (postprandial glycogen) and the newly synthesized glycogen:

$$\text{Preprandial hepatic glycogen (\mu mol/g dry wt)} = \text{postprandial glycogen} - \text{newly synthesized glycogen}$$

The sources of newly synthesized glycogen were resolved into direct and indirect pathway contributions on the basis that whereas all newly synthesized glycogen is enriched in position 2, glycogen derived via the indirect pathway is additionally enriched in position 5 (26). On this basis, the direct and indirect pathway contributions to newly synthesized glycogen were calculated from the ratio of positions 5 and 2 $^2$H enrichments (H5/H2) of glycogen as follows:

$$\text{Indirect pathway fraction (\%)} = 100 \times \frac{H5}{H2}$$

$$\text{Direct pathway fraction (\%)} = 100 - \text{indirect pathway fraction}$$

$$\text{Direct pathway contribution (\mu mol/g dry wt)} = \text{newly synthesized} \times \text{direct pathway fraction}/100$$

$$\text{Indirect pathway contribution (\mu mol/g dry wt)} = \text{newly synthesized} \times \text{indirect pathway fraction}/100$$

_Plasma glucose sources during overnight feeding._ The fraction of postprandial plasma glucose derived from endogenous G6P is enriched with $^3$H in position 2 to the same level as body water regardless of its origin (gluconeogenesis or glycogenolysis). Any dilution of position 2 enrichment is attributed to the appearance of absorbed unlabeled glucose originating from the diet. Thus, exogenous and endogenous fractions of plasma glucose were calculated as follows:

$$\text{Endogenous glucose fraction (\%)} = 100 \times \frac{H2/BW}{H5/H2}$$

$$\text{Exogenous glucose fraction (\%)} = 100 - \text{endogenous glucose fraction}$$

where $H2$ is the $^2$H enrichment of position 2.

The contribution of dietary absorption (nmol/l) was then calculated by multiplying the exogenous glucose fraction by the glycemia (mmol/l) measured at the end of the dark cycle.

The endogenous contribution to plasma glucose comprehends both gluconeogenic and nongluconeogenic sources. The gluconeogenic fraction was estimated as follows:

$$\text{Gluconeogenic fraction (\%)} = 100 \times \frac{H5}{H2/BW}$$

where $H5$ is the $^2$H enrichment of plasma glucose position 5.

The contribution of gluconeogenesis (mmol/l) was then calculated by multiplying the gluconeogenic fraction by the glycemia (mmol/l) measured at the end of the dark cycle.

The nongluconeogenic fraction of endogenous glucose (glycogenolysis and/or glucose/G6P/glucose cycling) was estimated as follows:

$$\text{Non gluconeogenic fraction (\%)} = 100 \times \left(\frac{H2 - H5}{H2/BW}\right)$$

where $H2$ and $H5$ are the plasma glucose $^2$H enrichments.

The contribution of nongluconeogenic endogenous sources (mmol/l) was then calculated by multiplying the nongluconeogenic fraction by the glycemia (mmol/l) measured at the end of the dark cycle.

_DNL during overnight feeding._ In the process of lipid biosynthesis, there is incorporation of $^2$H from the BW into the fatty acid molecule. Because the methyl groups of the fatty acids in the triglyceride (TG) molecule derive directly from acetyl-CoA (40), which is the building block in DNL, a simple method for the quantification of DNL consists in determining the $^2$H enrichment in the methyl group of TGs and relates that to the precursor enrichment in the presence of $^2$H$_2$O (10). Hence, the fraction of hepatic TG derived from DNL over the dark cycle was calculated as follows:

$$\text{DNL fraction (\%)} = 100 \times \frac{H5}{H2/BW}$$

where TG-methyl is the $^2$H enrichment of the triglyceride methyl and BW is the $^2$H enrichment of the body water.

**Statistics**

Data are presented as means ± SE. Statistical significance, defined as $P < 0.05$, was determined by one-way ANOVA followed by Fisher’s least significant difference (LSD) posttest.

**RESULTS**

Metabolic and Nutritional Parameters of STZ Rats Compared with Healthy Controls

Plasma metabolite levels and nutritional and endocrine parameters are shown in Table 1, and hepatic glycogen synthesis measurements are shown in Table 2. Plasma glucose and TG levels were significantly elevated in STZ rats compared with controls. STZ rats also experienced a significant loss of body weight, and postprandial endogenous insulin levels were ~10% that of healthy controls by the end of the study.

These alterations were accompanied by significant changes in hepatic carbohydrate and lipid metabolism during the nocturnal feeding period. Healthy animals had relatively abundant basal stores of glycogen at the beginning of the feeding period, and these levels increased by about one-third over the nocturnal feeding interval. As was observed previously (57), the synthesized glycogen was derived in approximately equal proportions from direct and indirect pathways (Table 2), as seen by a glycogen H5/H2 ratio of ~0.5 (Fig. 2). For STZ rats, basal glycogen levels were significantly less compared with those of nondiabetic controls. Whereas the fractional increase in hepatic glycogen levels following overnight feeding was significantly higher for STZ rats compared with healthy controls, postprandial hepatic glycogen levels attained only about one-third of control values. The synthesized glycogen was derived entirely via the indirect pathway, as demonstrated clearly by the $^2$H-NMR spectrum of the STZ rat in Fig. 2, where the H5/H2 ratio was ~1.0. By comparison, we previously observed a H5/H2 ratio of ~0.7 a few days after the STZ injection (57), suggest-
Table 1. Biochemical and physiological parameters determined during the insulin replacement study

<table>
<thead>
<tr>
<th>Parameter</th>
<th>STZ</th>
<th>I-SC</th>
<th>I-IP</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycemia, mg/dl</td>
<td>534 ± 22a</td>
<td>90 ± 10a</td>
<td>114 ± 22b</td>
<td>105 ± 2a</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>2.49 ± 31a</td>
<td>133 ± 10a</td>
<td>116 ± 10a</td>
<td>124 ± 7a</td>
</tr>
<tr>
<td>NEFA, mmol/l</td>
<td>0.58 ± 0.08a</td>
<td>1.52 ± 0.18b</td>
<td>1.15 ± 0.14b</td>
<td>0.31 ± 0.05a</td>
</tr>
<tr>
<td>Exogenous insulin, mU/l*</td>
<td>1.18 ± 1.15a</td>
<td>4.19 ± 1.02b</td>
<td>3.87 ± 1.40b</td>
<td>2.0 ± 0.50b</td>
</tr>
<tr>
<td>Endogenous insulin, µg/l</td>
<td>0.50 ± 0.20a</td>
<td>0.73 ± 0.12a</td>
<td>0.70 ± 0.19a</td>
<td>5.30 ± 1.10a</td>
</tr>
<tr>
<td>Hepatic triglycerides, µmol/g dry wt</td>
<td>319 ± 20a</td>
<td>301 ± 15a</td>
<td>287 ± 11a</td>
<td>212 ± 22b</td>
</tr>
<tr>
<td>EWAT, g/100 g</td>
<td>0.30 ± 0.07a</td>
<td>0.78 ± 0.09b</td>
<td>0.92 ± 0.14b</td>
<td>ND</td>
</tr>
<tr>
<td>Food intake, g/day</td>
<td>40 ± 1.1a</td>
<td>36 ± 2.4b</td>
<td>34 ± 0.3b</td>
<td>23 ± 1b</td>
</tr>
<tr>
<td>Water intake, ml/day</td>
<td>184 ± 14a</td>
<td>113 ± 15a</td>
<td>119 ± 10a</td>
<td>35 ± 3b</td>
</tr>
<tr>
<td>Weight evolution, AUC&lt;sub&gt;days 9-18&lt;/sub&gt;</td>
<td>229 ± 15a</td>
<td>280 ± 6b</td>
<td>268 ± 5b</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are means ± SE and are shown for streptozotocin (STZ) rats, STZ rats treated with insulin by subcutaneous (I-SC) or intraperitoneal (I-IP) injection, and nondiabetic controls. ND, not determined; NEFA, nonesterified fatty acids; EWAT, epidymal white adipose tissue; AUC, area under the curve. Glycemia and plasma triglyceride measurements were performed 2 h after the morning insulin injection on the 8th day of insulin treatment. Food and water consumption were measured in the last 24 h prior to euthanization. Weight evolution is expressed as the AUC in the weight vs. time plots from days 9 to 18 post-STZ. All of the other parameters correspond to postmortem measurements performed on tissue and blood samples collected in the morning. For each parameter, values not sharing a common superscripted letter are significantly different; statistical significance was accepted for P < 0.05, determined by 1-way ANOVA with Fisher’s least significant difference (LSD) posttest. *Human insulin assay.

Table 2. Hepatic glycogen synthesis during overnight feeding, with estimations of direct and indirect pathway contributions

<table>
<thead>
<tr>
<th>Overnight Hepatic Glycogen Excursions and Sources</th>
<th>Preprandial, µmol/g dry wt</th>
<th>Postprandial, µmol/g dry wt</th>
<th>Newly Synthesized</th>
<th>Direct Pathway</th>
<th>Indirect Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol/g dry wt</td>
<td>%Final</td>
<td>µmol/g dry wt</td>
<td>%Net synthesis</td>
<td>µmol/g dry wt</td>
</tr>
<tr>
<td>Controls</td>
<td>578 ± 55a</td>
<td>907 ± 84a</td>
<td>329 ± 86a</td>
<td>34 ± 7a</td>
<td>154 ± 46a</td>
</tr>
<tr>
<td>STZ</td>
<td>128 ± 19b</td>
<td>320 ± 51b</td>
<td>192 ± 35a</td>
<td>58 ± 3b</td>
<td>2 ± 1b</td>
</tr>
<tr>
<td>I-SC</td>
<td>444 ± 44a</td>
<td>680 ± 56b</td>
<td>236 ± 47a</td>
<td>34 ± 5a</td>
<td>129 ± 29a</td>
</tr>
<tr>
<td>I-IP</td>
<td>560 ± 80a</td>
<td>860 ± 65c</td>
<td>299 ± 64a</td>
<td>35 ± 7a</td>
<td>151 ± 32a</td>
</tr>
</tbody>
</table>

Data are means ± SE and expressed as µmol/g dry wt of liver and as percentages and are shown for nondiabetic controls, STZ rats, I-SC STZ rats, and I-IP STZ rats. For each parameter in each column, values not sharing a common superscripted letter are significantly different; statistical significance was accepted for P < 0.05, determined by 1-way ANOVA with Fisher’s LSD posttest.
any significant perturbation by the presence of exogenous human insulin. Thus, the I-SC and I-IP groups showed similar residual levels of endogenous insulin to the untreated STZ group.

Plasma glucose levels measured 2 h after either I-SC or I-IP administration were comparable with those of controls (Table 1), but by the end of overnight feeding (~12 h after insulin administration), glycemia had reverted to that of untreated STZ rats (Fig. 4). This indicates that euglycemia was only temporarily established by the insulin administration regimes. Accordingly, the gluconeogenic contribution to postprandial glucose in I-SC and I-IP groups remained similar to that of untreated STZ rats and well above control levels. However, I-SC and I-IP increased the contribution from glycolysis and/or glucose-G6P cycling to plasma glucose while decreasing the contribution from dietary absorption (Fig. 4).

The dyslipidemia of STZ rats was attenuated by both insulin therapy regimes, but the effects were more limited compared with those observed for hepatic glycogen. Whereas plasma TG levels were restored to those of controls, NEFA concentrations were elevated and were higher than those of untreated STZ rats. Both I-SC and I-IP therapies increased relative and absolute EWAT mass significantly compared with untreated STZ rats but failed to attenuate the elevated hepatic TG levels. Hepatic lipogenic activity, as gauged by the fractional DNL contribution to hepatic TG after overnight feeding, was increased significantly by insulin administration. However, these values remained significantly below those of non-diabetic control rats (Fig. 3).

The responses of hepatic glycogen, glucose, and lipid metabolism to insulin therapy were echoed by changes in activities (Table 3) and expression (Fig. 5) of key control enzymes...
for glycogen and lipogenesis. GK activities were restored to control levels corresponding with the complete recovery of direct pathway flux. PK and pentose pathway enzyme activities were partially restored, mirroring the partial recovery of DNL activity. In contrast to the key control enzymes of glycogenolysis and glycolysis, phosphofructokinase (PFK) activities were not modified significantly by insulin administration and remained significantly lower compared with those of controls. Changes in hepatic enzyme expression that were associated with insulin administration were partially correlated with the observed alterations in activities. GK expression was similar to control levels, but the increase relative to STZ rats was not significant. Meanwhile, expression of lipogenic enzymes matched or exceeded those of control rats. PFK and pentose pathway enzyme expressions were not significantly different between the four groups. Both I-SC and I-IP administration failed to normalize the tendency for elevated hepatic carnitine palmitoyltransferase Ia (CPT Ia) expression levels in STZ rats compared with controls; indeed, CPT Ia expression levels in I-SC and I-IP groups were significantly higher compared with controls. For the principal transcription factors involved in lipid and carbohydrate metabolism, sterol regulatory element-binding pro-

### DISCUSSION

In type 1 diabetes, a deficiency of insulin results in a remodeling of hepatic metabolic fluxes that contributes to the loss of whole body glucose and lipid homeostasis. The most widely used rodent model of type 1 diabetes involves the destruction of β-cell function by STZ administration. A high single dose of STZ causes rapid destruction of the β-cells, resulting in persistent hypoinsulinemia and hyperglycemia accompanied by weight loss (24, 64). By comparison, multiple low-dose injections of STZ act by a slower immune-mediated mechanism that may better mimic the development of human type 1 diabetes subjects (32, 35). Since we were interested specifically in the effects of insulin deficiency on hepatic carbohydrate and lipid metabolism, we chose the single STZ dosing model to rapidly establish insulin deficiency so that possible secondary metabolic effects of diabetes were minimized. In this model, hyperglycemia and dyslipidemia per se may also account for the changes in intrahepatic fluxes. For example, the glucose fatty acid cycle (50) establishes that fatty acids compete with glucose for oxidation, and thus high availability of fatty acids prevents overall glucose disposal, ultimately leading to hyperglycemia. It is well documented in both humans and rodents that hyperglycemia and hyperlipidemia per se cause insulin resistance, further contributing to the loss of metabolic control (3, 41, 51, 61). In our experiments, we addressed the overall metabolic profile that depends on the plasma levels of metabolic fuels (mainly glucose and fatty acids) as well as on endocrine and central control.

We evaluated how hepatic glycogen, glucose, and lipid metabolism were altered in rats following induction of diabetes and to what extent these changes were reversed by short-term I-SC and I-IP replacement. These observations were accompanied by assays for the activities and expressions of enzymes controlling intermediary metabolic fluxes, which can be modified both allosterically and transcriptionally by insulin. In our protocol, the interference with the natural feeding cycle and endogenous endocrine activities is minimal compared with traditional tracer infusion procedures. Therefore, we believe

### Table 3. Enzyme activities (mU/mg protein) determined in liver samples after overnight feeding

<table>
<thead>
<tr>
<th>Enzyme activities (mU/mg protein) determined in liver samples after overnight feeding</th>
<th>STZ</th>
<th>I-SC</th>
<th>I-IP</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycolysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GK</td>
<td>0.1 ± 0.0*</td>
<td>8.8 ± 0.3*</td>
<td>7.5 ± 0.4*</td>
<td>7.2 ± 1.0*</td>
</tr>
<tr>
<td>PFK</td>
<td>15.2 ± 1.0*</td>
<td>16.9 ± 0.7*</td>
<td>17.8 ± 1.2*</td>
<td>22.5 ± 0.5*</td>
</tr>
<tr>
<td>PK</td>
<td>79.0 ± 15.7*</td>
<td>463.6 ± 43.8b</td>
<td>333.6 ± 33.9e</td>
<td>814.7 ± 48.7e</td>
</tr>
<tr>
<td>Pentose pathway</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G6PDH</td>
<td>10.7 ± 0.6*</td>
<td>21.0 ± 3.2*</td>
<td>19.7 ± 1.7*</td>
<td>97.9 ± 12.1b</td>
</tr>
<tr>
<td>6PGDH</td>
<td>11.5 ± 0.7*</td>
<td>22.4 ± 1.6*</td>
<td>19.7 ± 0.8*</td>
<td>43.2 ± 3.1a</td>
</tr>
</tbody>
</table>

Data are means ± SE and are shown for STZ rats, I-SC STZ rats, I-IP STZ rats, and nondiabetic controls. GK, glucokinase; PFK, phosphofructokinase; PK, pyruvate kinase; G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase. For each enzyme activity, values not sharing a common superscript lettered are significantly different; statistical significance was accepted for P < 0.05, determined by 1-way ANOVA with Fisher’s LSD posttest.
that our method provides a more realistic description of postprandial hepatic intermediary metabolism.

In our experiments, we recreated a model of poorly controlled type 1 diabetes. Neither I-SC nor I-IP established glycemic control over the entire daily feeding and fasting cycle, and plasma glucose levels matched those of untreated STZ rats at the end of the dark period. The gluconeogenic contribution to plasma glucose levels at this time was comparable with untreated STZ rats, but a significant increase in contributions from glycogenolysis and/or glucose-G6P cycling was observed with concomitant reduction of the contribution from absorption. Given that gluconeogenic enzyme expression was not altered significantly, whereas GK activities were maintained and hepatic glycogen stores were preserved, the most likely mechanism for the fractional reduction in the absorptive contribution to plasma glucose levels is increased glucose-G6P cycling (37, 53) rather than increased glucose production from hepatic glycogenolysis. Reduction of food intake by insulin, although quite small in our experiments, would also contribute toward a decreased absorptive contribution.

Unlike gluconeogenesis, glycogen synthesis responded efficiently to insulin administration in STZ rats despite only intermittent restoration of glycemic control. Restoration of GK activity to control levels correlated with increased hepatic glycogen, in accord with the enhancement of glycogen synthesis in hepatocytes (44, 54) and diabetic rats (39) that were engineered to overexpress GK. Most significantly, insulin administration resulted in a complete recovery of direct pathway flux, which is highly dependent on insulin-mediated GK activation. These data bear interesting comparisons with studies of glycogen metabolism in type 1 diabetes patients following insulin therapy. For patients that established both excellent long-term and diurnal glycemic control by intensive insulin therapy, net hepatic glycogen synthesis rates and peak postprandial glycogen levels were restored to those of healthy controls (4). This was driven by increased indirect pathway activity, with direct pathway fluxes not being fully recuperated, suggesting that the postprandial induction of hepatic GK activity, on which the direct pathway depends heavily, may not have been fully restored by intensive insulin therapy. To our knowledge, the effects of insulin administration on hepatic GK expression and activities have not been reported for type 1 diabetes subjects; therefore, the relationship between insulin administration, GK activation, and direct pathway flux in humans remains unknown. The recuperation of direct pathway glycogen synthesis in our model may reflect species differences, with higher mammals having more complex regulation networks arising from evolutionary adaptations. It should also be noted that our metabolic assessments were carried out in STZ rats at an early diabetic stage, whereas human studies are performed in young adults several years after diagnosis of type 1 diabetes, which is established typically during childhood. At an early diabetic condition, it was shown that STZ rats were able to replenish the hepatic glycogen stores after exercise in the absence of food intake (16), thereby providing evidence that glycogen synthesis flux (probably via direct pathway activity) is still responsive in STZ rats at least early in the diabetic condition.

Besides a direct action in the liver, insulin also affects hepatic metabolism indirectly through the insulin-brain-liver axis, with intracerebroventricular administration of insulin resulting in the inhibition of hepatic glucose production in rats (45). However, such studies for assessing central insulin action are performed under well-controlled experimental conditions in which the liver is deprived from endogenous insulin and glucagon signals. Hence, the physiological role for central insulin in the control of hepatic glucose metabolism is still not clearly established (17, 28, 48). It has been argued that, under physiological conditions, central insulin action is very minor when compared with its noncentral effects (17), but it may gain importance in type 1 diabetes when insulin is delivered to the systemic circulation with consequent loss of the physiological hepatic gradient (28). It was shown in dogs that insulin delivery to arteries that supply the brain in the presence of relative hepatic insulin deficiency reduced glucose output by diverting the gluconeogenic flux from glucose production to glycogen synthesis (49). This is concordant with the observation that restoration of tight glycemic control and postprandial hepatic glycogen repletion rates in type 1 diabetes, through intensive systemic insulin therapy, were associated with increased indirect pathway fluxes (4).

In principle, the use of human insulin to treat the STZ rats coupled with ELISA assay kits that are specific for rat and human insulin allow the plasma levels of endogenous and administered insulin to be resolved. In practice, we recorded...
high background levels of human insulin from animals that did not receive any human insulin (both STZ rats and controls). Ackermans et al. (1) also found high cross-reactivities for assays of human insulin in rat plasma and determined that the values of exogenous insulin depended on the source of exogenous insulin, the sample matrix, and the type of assay kit. Under these conditions, they concluded that although absolute exogenous insulin concentrations cannot be well determined, it is still possible to relate changes in measured exogenous insulin concentrations to metabolic outcomes.

In our experiments, the STZ rats treated with I-SC recapitulated the systemic insulin delivery in type 1 diabetes, whereas with the I-IP we sought to emphasize the direct action of insulin in the liver. In either case, hepatic glucose production was not suppressed appropriately, most likely because insulin delivery was poorly coupled to feeding activity. Even so, we anticipated that hepatic metabolic fluxes of STZ rats would respond better to I-IP vs. I-SC, since I-IP better recreates the physiological portal-peripheral insulin gradient (47). Postprandial glycogen was lower in I-SC than in controls, whereas I-IP resulted in its normalization to control levels. Nevertheless, hepatic GK expression and activities responded similarly to I-SC and I-IP treatments. It is possible that the interval between the last insulin injection and liver harvesting blunted some differential effects of I-IP vs. I-SC administration. Mason et al. (34) reported that, with continuous insulin diffusion pumps, I-IP resulted in increased stimulation of GK activity relative to I-SC and even nondiabetic controls. They also observed that, although lipogenic enzyme activities were enhanced to the same extent by both routes (33), I-IP improved glyceremic control compared with I-SC (8, 42). Moreover, compared with I-SC, I-IP was better at suppressing endogenous glucose production and also decreased peripheral hyperinsulinemia (34, 62). In pigs, portally infused insulin was also shown to provide better control of hepatic glucose output compared with subcutaneous infusion (6). Thus, portal insulin may be important in attenuating hepatic glucose production by promoting hepatic GK activity and glycogenesis. STZ rats overexpressing a constitutively active form of liver glycogen synthase resulting in normalized hepatic glycogen levels were also shown to have improved glyceremic control despite low insulin and GK expression (52), suggesting that hepatic glycogen levels may influence glyceremic control independently of insulin and GK. Moreover, increasing the glycogen pool in the liver results in the inhibition of glycogen synthesis while preserving hepatic glucose uptake during the hyperglycemic hyperinsulinemic state (65). Under these circumstances, hepatic G6P conversion to lactate and CO2 was increased proportionally in the short-term period of the experiment. Such a shift in glucose disposal from glycogen synthesis to glycolysis supports an increase of the lipogenic pathway in the long term, further emphasizing the role of hepatic glycogen stores in the control of intrahepatic glucose fluxes. In our current study, the restoration of hepatic glycogen levels accomplished by the insulin therapies in the STZ rat was indeed associated with a recuperation of de novo lipogenic activity.

Following induction of diabetes by STZ, there was a notable reduction in DNL contribution to hepatic triglyceride levels. This is consistent with previous observations of reduced hepatic triglyceride labeling from 3H2O for both perfused and in situ livers obtained from STZ rats compared with nondiabetic controls (18, 29). Insulin deficiency also impaired lipid uptake into adipose tissue in the STZ rats, as demonstrated by the reduced EWAT content and elevated plasma triglyceride levels. Presumably, triglycerides that were not cleared by the adipocytes accumulated in ectopic sites, including liver. The elevation of hepatic triglyceride as a consequence of endogenous lipid redistribution, rather than of unregulated hepatic de novo production, was further demonstrated by the decreased expression levels of DNL enzymes. In this setting, we anticipated that the NEFA flux from adipose tissue lipolysis would be increased, in agreement with the reduced insulin levels. The similar plasma NEFA concentration between STZ rats and controls may be explained by the depleted lipid stores in the adipose tissue. In accord with this, the EWAT content of STZ rats represented only ~0.3% of the animals’ body weight compared with 0.6–1.2% reported for healthy controls (11, 58, 66).

Plasma NEFA concentrations were higher in STZ rats undergoing insulin replacement over the untreated STZ group. This is contrary to previous reports, where NEFA levels of diabetic rodents were reduced by insulin administration (12, 62), and at first glance also seems to contradict the higher EWAT mass for insulin-treated compared with untreated STZ animals. This paradox may reflect the partial restoration of metabolic control by the insulin replacement procedure where triglyceride storage is initially active after insulin administration, but lipolysis is reasserted once insulin levels decline. Compared with untreated animals, insulin-treated animals store more triglyceride in adipose tissue, but when insulin levels decline their larger triglyceride pools are able to sustain a higher release of NEFA.

With higher availability of circulating NEFA, increased contributions from de novo production, and presumably an attenuated VLDL secretion, it was surprising that the hepatic TG content of insulin-treated STZ rats was not increased over that of the untreated group. One possibility is that hepatic β-oxidation activity remained upregulated despite insulin treatment, as suggested by the increased hepatic CPT Ia expression levels, such that the higher lipid influx was balanced by increased fatty acid oxidation. This would also imply a loosening of the tight reciprocal control that normally exists between DNL and mitochondrial β-oxidation, where CPT Ia activity is inhibited by malonyl-CoA generated during lipogenesis, thereby preventing simultaneous synthesis and oxidation of hepatic fatty acids. In hepatocytes that overexpressed CPT Ia, inhibition of palmitate oxidation by malonyl-CoA was shown to be significantly attenuated (2). Moreover, in STZ rats the sensitivity of CPT Ia inhibition to malonyl-CoA was decreased and only partially restored by high insulin doses (20).

Insulin deficiency is the hallmark of type 1 diabetes, and therefore, therapy has been based traditionally on insulin replacement regimens. However, in past years, diabetes research brought into light other endocrine players, like glucagon or leptin. For example, the deletion of the glucagon receptor in mice is sufficient to prevent the development of type 1 diabetes symptoms after STZ (27) and aloxan treatments (63). Interestingly, one proposed mechanism for the leptin-induced normalization of blood glucose is the suppression of glucagon secretion (19, 63). Hence, there is increasing evidence supporting the critical role of glucagon in diabetes pathology (13). We did not measure glucagon levels in our experiments, but the STZ
rat model is known to display relative hyperglucagonemia. Even if glucagon levels are normal (15, 62) there is no insulin to oppose its actions, leading to a permanent and poorly controlled catabolic state characterized by the stimulation of endogenous glucose production and lipid mobilization. In our study, because we used short-acting insulin in twice daily injections, STZ rats receiving treatment would remain relatively hyperglucagonemic with increasing time after the injections. Therefore, we can speculate that insulin would promote carbohydrate and lipid storage in the early period after its administration, but afterward lipolysis and glycolysis would again be activated by glucagon. Indeed, and as discussed above, parallel with increased EWAT mass we detected high values of circulating NEFA from lipolysis. Enhanced hepatic DNL was also present along with increased β-oxidation. On the other hand, glycolysis appears to have been inhibited efficiently even in face of the poor insulinization, suggesting that glycogen fluxes can be regulated normally even in the presence of uncorrected hyperglucagonemia, presumably via GK activity.

Type 1 diabetes patients currently rely on peripheral insulin administration for glycemic control. Although intensive therapy should be sufficient to overcome glucagon-dependent catabolism, it is unable to recreate the high portal insulin levels of healthy subjects. This may limit the metabolic performance of insulin-treated diabetic patients, particularly in the case of hepatic-specific actions such as direct pathway glycogen synthesis. Furthermore, inappropriate glycogen levels can contribute to the deregulation of intrahepatic glucose fluxes.

In conclusion, we applied 2H2O to obtain a hepatic metabolic profile of naturally feeding animals and characterized significant derangements in hepatic glycogen, glucose, and TG metabolism in rats that had been made diabetic by STZ injection. Twice-daily insulin administration only partially restored glycemic control, but hepatic glycogen levels and overnight synthesis via direct and indirect pathways were fully recovered. These observations were associated with a recovery of the lipogenic pathway in the liver.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


