Acute inhibition of hepatic glucose-6-phosphatase does not affect gluconeogenesis but directs gluconeogenic flux towards glycogen in fasted rats

A pharmacological study with the chlorogenic acid derivative S4048

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Effects of acute inhibition of glucose-6-phosphatase (G6Pase) activity by the chlorogenic acid derivative S4048 on hepatic carbohydrate fluxes was examined in isolated rat hepatocytes and in vivo in rats. Fluxes were calculated using tracer dilution techniques and mass isotopomer distribution analysis in plasma glucose and urinary paracetamol-glucuronide after infusion of [U-13C]-glucose, [2-13C]-glycerol, [1-2H]-galactose and paracetamol. In hepatocytes, glucose-6-phosphate (G6P) content, net glycogen synthesis and lactate production from glucose and dihydroxyacetone increased strongly in the presence of S4048 (10 μM). In livers of S4048-treated rats (0.5 mg kg⁻¹ min⁻¹; 8h) G6P content increased strongly (+440%) and massive glycogen accumulation (+1260%) was observed in periportal areas. Total glucose production was diminished by 50%. The gluconeogenic flux to G6P was unaffected, i.e. 33.3 ± 2.0 μmole kg⁻¹ min⁻¹ in control and S4048-treated rats, respectively. Newly synthesized G6P was redistributed from glucose production (62 ± 1 % vs 38 ± 1 %; p<0.001) to glycogen synthesis (35 ± 5 % vs 65 ± 5 %; p<0.005) by S4048. This was associated with a strong inhibition (-82%) of the flux through glucokinase (GK), an increase (+83%) of the flux through glycogen synthase (GS) while the flux through glycogen phosphorylase (GP) remained unaffected. In livers from S4048-treated rats mRNA levels of genes encoding G6P hydrolase (~ 9-fold), G6P translocase (~ 4-fold), GS (~ 7-fold) and L-type pyruvate kinase (~ 4-fold) were increased, whereas GK expression was almost abolished. In accordance with unaltered gluconeogenic flux, expression of the gene encoding phosphoenolpyruvate carboxykinase was unaffected in the S4048-treated rats. Thus, acute inhibition of G6Pase activity by S4048 elicited: 1) a re-partitioning of newly synthesized G6P from glucose production into glycogen synthesis without affecting the gluconeogenic flux to G6P, 2) a cellular response aimed at maintaining cellular G6P homeostasis.
INTRODUCTION

Glucose-6-phosphate (G6P) plays a pivotal role in hepatic carbohydrate metabolism both as a metabolite and as a signaling compound. G6P is the shared intermediate of gluconeogenesis (Figure 1, I + IV) and glycogenolysis (Figure 1, II) and is formed by glucokinase (GK)-mediated glucose phosphorylation (Figure 1, III). G6P provides the substrate for glucose production by the liver, via hydrolysis by glucose-6-phosphatase (G-6-Pase; Figure 1, IV). It serves as substrate for glycolysis (Figure 1, V) and is the obligatory precursor for the synthesis of glycogen via UDP-glucose (Figure 1, VI). Partitioning of newly synthesized G6P into glucose production, degradation via glycolysis or storage as glycogen offers modes of autoregulating hepatic glucose production without affecting the rate of gluconeogenesis. G6P stimulates the activity of glycogen synthase (GS) b and of GS phosphatase (1). G6P and/or its pentose-phosphate derivative xylulose-5-phosphate (X5P) act as signaling compound in the control of gene expression (see ref. 2 for a review). Recent data show that the effect of insulin on gene expression of hepatic enzymes involved in carbohydrate metabolism critically depends on concomitant intracellular metabolism of glucose (3,4), supporting a sequence of events starting with the direct induction of GK expression by insulin. Enhanced activity of GK results in increased intracellular concentrations of G6P and/or X5P. This appears to be essential in the action of insulin on the stimulation of expression of genes involved in glucose production, glycolysis and lipogenesis, e.g., hydrolytic subunit of glucose-6-phosphatase (G6PH), glucose transporter type 2 (GLUT-2), liver-type pyruvate kinase (L-PK), ATP-citrate lyase, acetyl-CoA carboxylase and fatty acid synthase (see ref. 2 for a review).

Since G6P participates in so many reactions in hepatic glucose metabolism, the relationship between hepatic glucose production and gluconeogenesis in vivo is very complex. A major problem in studying G6P partitioning in vivo resides in the choice of precursor, label and...
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isotopic model. In earlier studies, substrates labeled with $^{14}$C or $^{13}$C have been applied followed by determination of positional isotopomer distribution in either plasma glucose (5) or in urinary N-phenylacetylglutamine (6). Relative gluconeogenic fractions obtained in this way were converted into absolute rates of gluconeogenesis by multiplying with the plasma glucose turnover rate. With this method the contribution of a particular substrate to the gluconeogenic flux directed into plasma glucose can be calculated. More recent methods estimate gluconeogenic flux from precursors directed to plasma glucose; these methods comprise $^2$H incorporation into specific positions in plasma glucose from $^2$H$_2$O (7) or incorporation of [2-$^{13}$C]-glycerol into mass isotopomers of plasma glucose (8,9). The development of an improved isotopic model based on the last method allows for the calculation of flux rates of newly synthesized G6P into plasma glucose as well as into glycogen (10). In the latter model, incorporation of [2-$^{13}$C]-glycerol is measured in plasma glucose and urinary paracetamol-glucuronide (p-GlcUA), as markers of 2 major metabolic routes of G6P, e.g. hepatic glucose production and glycogen synthesis via UDP-glucose, respectively (Figure 1, I + IV and I + VI, respectively). The obtained fractional contributions for plasma glucose and UDP-glucose (via p-GlcUA), respectively, are subsequently converted in absolute rates of gluconeogenic flux, directed to each of the compounds, by multiplying with the rates of appearance of plasma glucose and UDP-glucose (via p-GlcUA), respectively. After correction for exchange of newly synthesized G6P between plasma glucose and glycogen, via UDP-glucose, the total gluconeogenic flux into G6P is obtained. It should be realized, however, that the gluconeogenic flux into G6P thus obtained represents a minimal estimate, since the flux of G6P into glycolysis (Figure 1, V) is not considered in this isotopic model.

Using this isotopic model we have studied the effects of acute pharmacological inhibition of G6Pase in vitro and in vivo on the rate of gluconeogenesis and on the partitioning of G6P. Recently, a novel class of chlorogenic acid derivatives has been developed that inhibit G6Pase activity by blocking glucose-6-phosphate translocase (G6PT) (11). In experiments in anaesthetized rats and perfused rat livers, it was demonstrated that these compounds inhibit hepatic glucose production and lower blood glucose concentration in a dose-dependent way (12,13). We addressed the following questions: 1) does inhibition of hepatic glucose production by G6PT blockade result in an inhibition of gluconeogenic flux into G6P and/or a change in the partitioning of G6P and 2) does inhibition of G6PT acutely influence gene expression of enzymes involved in G6P metabolism.

EXPERIMENTAL PROCEDURES

Materials. [1-$^2$H]-galactose (99.6% $^2$H APE) was purchased from Isotec, Inc (Miamisburg, OH, USA) and [2-$^{13}$C]-glycerol (99.9% $^{13}$C APE), and [U-$^{13}$C]-glucose 99.9% $^{13}$C APE) was purchased from CIL, Inc (Andover, MA, USA). All chemicals were pro analysis grade. Infusates were freshly made and sterilized by the Hospital Pharmacy the day before an experiment.

In vitro experiments. Hepatocyte were isolated al from 20-24-h-starved male Wistar rats (250gr) by ex situ liver perfusion with collagenase (14). Incubations of freshly isolated hepatocytes (5-10 mg dry mass/ml) were carried out at 37 °C in closed 25-ml plastic scintillation vials containing 2 ml in Krebs-Henseleit bicarbonate medium plus 10 mM sodium HEPES (pH 7.4) and, where indicated, either 10 mM dihydroxyacetone or 20 mM glucose as substrate; the gas phase was 95% O$_2$ and 5% CO$_2$ v/v.
**In vivo experiments.** Male Wistar rats (275 ± 14 gr) were bred at the Central Animal Laboratory, University of Groningen (The Netherlands). The animals were housed in plexiglas cages (25 x 25 x 30 cm), with a controlled light-dark regime (12 hr dark and 12 hr light) and had free access to water and food (RMH-B, Hope Farms BV, Woerden, The Netherlands). One week before the experiment the animals were equipped with two permanent heart catheters, one for infusion and one to draw blood samples, as described by Kuipers et al (15). Twenty-four hours before the start of the experiments, food was removed but the animals had still free access to water.

On the day of the experiment, the animals were placed in metabolic cages, which allowed continuous collection of urine. The animals were infused with [U-\(^{13}\)C] glucose (1.0 ± 0.1 µmole kg\(^{-1}\) min\(^{-1}\) ), [2-\(^{13}\)C]-glycerol (9.2 ± 0.5 µmole kg\(^{-1}\) min\(^{-1}\) ), [1-\(^{2}\)H]-galactose (4.7 ± 0.2 µmole kg\(^{-1}\) min\(^{-1}\) ), paracetamol (total dose: 212 ± 10 mg kg\(^{-1}\) ) and, where indicated, S4048 (total dose: 265 ± 13 mg kg\(^{-1}\) ) in a sterile isotonic solution consisting of phosphate buffered saline (pH 7.2) with DMSO (6.1% v/v). Blood samples (200 µl) were drawn before the start of the infusion and 3, 6, 7 and 8 h thereafter. Timed urine samples were collected at hourly intervals. The blood samples were collected in heparin-containing tubes and centrifuged immediately. Plasma and urine samples were stored at –20 °C until analysis. At the end of the experiment, the animals were anaesthetized with pentobarbital, a large blood sample was taken by heart puncture and the liver was excised, weighed and parts were frozen immediately in liquid N\(_2\).

**Metabolite Assays.** Glucose and lactate in hepatocyte incubations were determined in HClO\(_4\)-extracted, KOH-neutralized samples with ATP, NADP\(^{+}\), hexokinase and G6P dehydrogenase (glucose) and with NAD\(^{+}\) and lactate dehydrogenase (16). The glycogen content of hepatocytes was measured as follows. Aliquots of cells were diluted with 4 vol of ice-cold 0.9% NaCl with 10 mM MOPS (pH 7.4) and centrifuged. After removal of the clear supernatant the pellets were dissolved in 0.1 M KOH and heated for 40 min at 85 °C. The solution was acidified to pH 4.5 with acetic acid (3M) and centrifuged to remove the protein. To 100 µl of the supernatant 0.14 U amyloligosidase was added and the mixture was incubated for 2 h at 40 °C. The glucose formed was measured fluorometrically as described (16). Background glucose was measured in identically treated samples, without addition of amyloglucosidase (16). For measurement of intracellular G6P, an aliquot of the cell suspension was diluted with 4 volumes ice-cold 0.9% NaCl plus 10 mM MOPS (pH 7.4) and centrifuged for 1 sec in a microcentrifuge. The cell pellet was immediately extracted with HClO\(_4\) (4%, w/v) and the precipitate was neutralized with a mixture of 2 M KOH and 0.5 M MOPS. G6P was determined fluorimetrically with NADP\(^{+}\) and G6P dehydrogenase (16). Samples for measurement of glycogen and G6P of liver tissue were prepared by extracting liquid N\(_2\)-cooled liver powder (about 100 mg wet weight) with either 1 ml 0.1 M KOH (glycogen) or HClO\(_4\) (4% w/v; G6P); this was then followed by the same procedure as described above for hepatocytes. Plasma insulin was determined by a radio immunoassay RI-13K (Linco Research, Inc., St Charles, MO, USA). Plasma glucose concentration was determined enzymatically by use of the Beckmann glucose analyzer II (Beckmann Instruments, Palo Alto, CA, USA).

**Liver histology.** To visualize glycogen deposition in the liver staining with PAS was performed on 4 µm thick slices from frozen livers excised from the studied rats according to standard procedures.

**Hepatic mRNA levels.** Total RNA was isolated from approximately 30 mg of liver tissue using Trizol-method (GIBCO, Paisley, United Kingdom) followed by the SV Total RNA
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Isolation System (Promega, Madison, WI, USA) according to the protocols provided by the manufacturer. Isolated total RNA was converted to single stranded cDNA by a RT procedure with M-Mulv-RT (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer’s protocol. For PCR amplification studies, amounts of cDNA corresponding to ~30 ng of RNA were amplified with Taq DNA polymerase (Boehringer Mannheim, Mannheim, Germany) and the appropriate forward and reverse primers (GIBCO, Paisley, United Kingdom), essentially according to manufacturer’s protocols and optimized for the particular amplification cycler used. In the same experiments, calibration curves were run on serial dilutions of a 4x concentrated cDNA solution, resulting in a series containing 4x, 2x, 1x, 0.5x, 0.125x, 0.062x, and 0.031x of the cDNA present in the assay incubation. Gel electrophoresis of both assay and calibration incubations were done simultaneously. All gels were photographed with an Image Master VDS system (Pharmacia, Upsalla, Sweden) and intensities were quantified by video-scanning densitometry, using the software program Image Master 1D Elite 3.0 (Pharmacia, Upsalla, Sweden). All quantified intensities of experimental samples were within the linear part of the calibration curves. The following primer sequences were used: G6PH forward primer: ACT TTG GGA TCC AGT CGA CT and reverse primer: ACA GCA ATG CCT GAC AAG AC; G6PT forward primer: ATG AGA TCG CTC TGG ACA AG and reverse primer: TTC GGA GTC CAA CAT CAG CA; GK forward primer: GTG GGC TTC ACC TTC TCC TT and reverse primer: TCA CCA TGT CCA CCA CAT CC; GLUT2 forward primer: GGA TCT GCT CAC ATA GTC AC and reverse primer: TCT GGA CAG AAG AGC AGT AG; GS forward primer: CCA ATT CCA TGA ATG GCA GG and reverse primer: GCC TGG ATA AGG ATT CTA GG; GP forward primer: GAG ACT ACA TTC AGG CTG TG and reverse primer: CTA GCT CAC TGA AGT CCT TG; L-PK forward primer: TAC ATT GAC GAC GGG CTC AT and reverse primer: ATG CTC TCC AGC ATC TGT GT; PEPCK forward primer: GCC AGG ATC GAA AGC AAG AC and reverse primer: CCA GTT GAC CAA AGG CT and β-actin forward primer: AAC ACC CCA GCC ATG TAC G and reverse primer: ATG TCA CGC ACG ATT TCC C.

**MASS ISOTOPOMER DISTRIBUTION ANALYSIS**

*Isolation and derivatisation of plasma glucose.* Fifty µl of plasma was deproteinized by adding 500 µl ice-cold ethanol. The mixture was placed on ice for 30 minutes and then centrifuged. The supernatant was divided into two equal portions. Each portion was transferred to a reaction vial with Teflon-faced cap and dried by evaporation at 60 ºC under N₂. After cooling down, the first portion was derivatized to glucose-penta-acetate by adding 150 µl pyridine/acetic anhydride 1:2 (v/v) to the dry residue and incubating for 30 minutes at 60 ºC, followed by drying at 60 ºC under N₂. The dry residue was dissolved in 500 µl ethylacetate and transferred to an injection vial. The second portion was derivatized to glucose-aldonitril-penta-acetate by adding 50 µl pyridine containing hydroxylamine (2%; v/v) to the dry residue and incubating for 45 minutes at 100 ºC. After cooling, 100 µl acetic anhydride was added and the mixture was incubated for another 30 minutes at 60 ºC, followed by drying at 60 ºC under N₂. The dry residue was dissolved in 500 µl ethylacetate and transferred to an injection vial.

*Isolation and derivatisation of paracetamol-glucuronide (p-GlcUA).* For isolation of p-GlcUA urine samples (0.5 ml) were centrifuged to remove any debris and the supernatant was injected onto a Nucleosil 7C18 SP250/10 column. The HPLC system consisted of a Milton Roy CM4000 pump and a Milton Roy SM4000 variable wavelength ultraviolet detector (Interscience, Breda, The Netherlands). Millennium software (Waters, Etten Leur, The
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Netherlands) was used for peak integration. To achieve baseline separation of the p-GlcUA peak, a 2% buffer gradient program was applied consisting of buffer A containing 2% (w/v) ammonium formate in water (pH 4.8) and buffer B containing 40% CH₃CN in water. The program started with 100% A and 0% B at 3.3 ml/min. At 10.7 minutes the composition was changed to 92.5% A and 7.5% B within 0.1 minute and at 20 minutes buffer B was increased to 100% within 2 minutes. Under these conditions, the p-GlcUA peak eluted at 18.7 minutes, in a volume of 1.2 ml. The collected fraction was divided into two portions of 0.6 ml each. Each fraction was transferred to a Teflon-capped reaction vial and both fractions were dried at 115 °C under N₂. After cooling, p-GlcUA was derivatized to its tetraTMS-ethyl-ester by adding 400 µl ethanol/acetylchloride, 10:1 (v/v) to the dry residue and incubating for 45 min at room temperature, followed by drying at 60 °C under N₂. To the dry residue 200 µl BSTFA/pyridine/TCMS, 5:1:0.07 (v/v) was added and incubating for 120 min at 90 °C. After drying 1 ml ethylacetate was added. The dry residue of the second fraction was oxidized to saccharic acid by reacting with 35 µl sodium nitrite (0.4 g/ml water) and 70 µl nitric acid (32.5% in water) at 130 °C for 25 minutes, followed by drying at 60 °C under N₂. After cooling, saccharic acid was derivatized to its tetra-acetate-diethyl-ester by adding 400 µl ethanol/acetylchloride 10:1 (v/v) and incubating for 45 minutes at room temperature, followed by drying at 60 °C under N₂. To the dry residue 150 µl pyridine/acetic anhydride, 1:2 (v/v), was added and incubating for 30 minutes at 60 °C, followed by drying at 60 °C under N₂. The dry residue was dissolved in 50 µl ethylacetate and transferred to an injection vial.

**GC-MS procedures.** All samples were analyzed by gas chromatography-mass spectrometry. Derivatives were separated on a HP 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) using an AT-5 20 m x 0.18 mm ID (0.4 µm film thickness) capillary column (Alltech, Breda, the Netherlands). The GC temperature profile for p-GlcUA tetra-TMS-ethyl-ester was as follows: the initial temperature was 250 °C for 2 minutes and rose then to 280 °C at a rate of 25 °C/min. The column was held at 280 °C for 10 min. The compound eluted at 10.0 min. The m/z 331-337 ions, representing the m₀-m₆ mass isotopomers, were monitored in electron impact mode. The same GC temperature profile was used for glucose-penta-acetate, glucose-aldonitril-penta-acetate and saccharic acid tetra-acetate-diethyl-ester derivatives. The initial temperature was 80 °C for 1 min and rose then to 280 °C at a rate of 20 °C/min. The column was held at 280 °C for 5 min. The compounds eluted at 8.1, 10.6 and 10.9 min, respectively. Chemical ionization with methane was used. The ions monitored for glucose-penta-acetate were m/z 331-337, corresponding to the m₀-m₆ mass isotopomers. The ions monitored for glucose-aldonitril-penta-acetate were m/z 328-334, corresponding to the m₀-m₆ mass isotopomers. The ions monitored for saccharic acid tetra-acetate-diethyl-ester were m/z 375-381, corresponding to the m₀-m₆ mass isotopomers. The accuracy of the measurement was checked by injection of a standard sample after every eight experimental samples. The series were rejected when the reproducibility of the measurement of the standard sample was less than 1% for m₀ and less than 2% for m₁ and m₂. To check the range of intensities at the m/z values, which allows for reproducible analyses, dilution series were routinely made.

**Calculations.** Metabolic fluxes at steady state were calculated essentially according to Hellerstein et al. (10). The isotopic model of hepatic glucose metabolism is very similar to the one shown in Figure 1, with the exception that glycolysis (Figure 1, V) is absent. In this model 2 metabolic pathways give rise to plasma glucose and hepatic UDP-glucose formation, i.e., the gluconeogenic flux to G6P (Figure 1, I) and glycogenolysis (Figure 1, II). At steady state glycogenesis (Figure 1 VI) equals the formation of UDP-glucose (17, 18).
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Rate of appearance and recycling. Rates of appearance of glucose into the plasma glucose pool (Ra(glc)) and into the UDP-glucose pool (Ra(UDPglc)) via p-GlcUA were calculated by isotope dilution (19):

\[
Ra(glc) = \left\{ \frac{\text{MPE}(\text{glc};m_6)_{\text{infuse}}}{\text{MPE}(\text{glc};m_6)_{\text{plasma}}} - 1 \right\} \times \text{infusion}(\text{glc};m_6),
\]

[1]

in which MPE(\text{glc};m_6)_{\text{infuse}} and MPE(\text{glc};m_6)_{\text{plasma}} are the mole percent enrichments of \([U-^{13}C]\)-glucose in the infusate and plasma, respectively, and infusion(\text{glc};m_6) is the infusion rate of \([U-^{13}C]\)-glucose and

\[
Ra(UDPglc) = \left\{ \frac{\text{MPE}(\text{gal};m_1)_{\text{infuse}}}{\text{MPE}(\text{pGlcUA};m_1)_{\text{urine}}} - 1 \right\} \times \text{infusion}(\text{gal};m_1),
\]

[2]

in which MPE(\text{gal};m_1)_{\text{infuse}} and MPE(\text{pGlcUA};m_1)_{\text{urine}} are the mole percent enrichments of \([1-^2H]\)-galactose in the infusate and p-GlcUA in urine, respectively, and infusion(\text{gal};m_1) the infusion rate of \([1-^2H]\)-galactose. Ra(UDPglc) was calculated based on the assumption of a constant and complete entry of galactose into the hepatic UDP-glucose pool and that the label distribution in urinary p-GlcUA reflects the label distribution in UDP-glucose. The contribution of recycling should be added to these rates of appearance to obtain the total rates of appearance (10,20). For the calculation of recycling 2 correction factors are introduced (10): the fractional contribution of plasma glucose to UDPglucose formation c(glc)

\[
c(glc) = \frac{\text{MPE}(\text{pGlcUA};m_6)_{\text{urine}}}{\text{MPE}(\text{glc};m_6)_{\text{plasma}}},
\]

[3]

in which MPE(\text{pGlcUA};m_6)_{\text{urine}} and MPE(\text{glc};m_6)_{\text{plasma}} are the mole percent enrichments of urinary p-GlcUA and plasma glucose, respectively, during an infusion of \([U-^{13}C]\)glucose and the fractional contribution of UDP-glucose to plasma glucose formation c(UDPglc)

\[
c(UDPglc) = \frac{\text{MPE}(\text{glc};m_1)_{\text{plasma}}}{\text{MPE}(\text{pGlcUA};m_1)_{\text{urine}}}
\]

[4]

in which MPE(\text{glc};m_1)_{\text{plasma}} and MPE(\text{pGlcUA};m_1)_{\text{urine}} are the mole percent enrichments of urinary p-GlcUA and plasma glucose, respectively, during an infusion of \([1-^2H]\)-galactose. Recycling of glucose (r(glc)) and UDP-glucose (r(UDPglc)) were calculated according to:

\[
r(glc) = \left( \frac{c(glc)}{1-c(glc)} \right) \times Ra(glc)
\]

[5]

which is also a measure of glucose/G6P cycling (10, 20) and

\[
r(UDPglc) = \left( \frac{c(UDPglc)}{1-c(UDPglc)} \right) \times Ra(UDPglc)
\]

[6]

Total rates of appearance of glucose into the plasma glucose pool (totalRa(glc)) and into the hepatic UDP-glucose pool (totalRa(UDPglc)) were calculated according to:

\[
totalRa(glc) = Ra(glc) + r(glc)
\]

[7]
Rate of gluconeogenesis. The fractional gluconeogenic flux into both plasma glucose (f(glc)) and hepatic UDP-glucose (f(UDPglc); as measured in urinary p-GlcUA) were calculated by MIDA as described in detail elsewhere (8,21). The gluconeogenic flux into plasma glucose (GNG(glc)) and into UDP-glucose (GNG(UDPglc)) were calculated according to:

\[ \text{GNG(glc)} = f(glc) \times \text{totalRa(glc)} \]  
\[ \text{GNG(UDPglc)} = f(UDPglc) \times \text{totalRa(UDPglc)}. \]

Total gluconeogenic flux (totalGNG) is the sum of both components corrected for the exchange of label between the plasma glucose and hepatic UDPglucose pools (10):

\[ \text{totalGNG} = (1-c(glc)) \times \text{GNG(glc)} + (1-c(UDPglc)) \times \text{GNG(UDPglc)}. \]

Glycogenolysis. The contribution of glycogenolysis to glucose formation (GLY(glc)) and to UDP-glucose formation (GLY(UDPglc)) were calculated according to:

\[ \text{GLY(glc)} = (1-f(glc)) \times \text{totalRa(glc)}, \]
\[ \text{GLY(UDPglc)} = (1-f(UDPglc)) \times \text{totalRa(UDPglc)} - c(glc) \times \text{totalRa(UDPglc)}. \]

in which the contribution of glycogenolysis to the total rate of appearance of glucose in plasma is equal to the part, which does not derive from gluconeogenesis and in which c(glc) x totalRa(UDPglc) is the flux of plasma glucose into the hepatic UDP-glucose pool. In contrast to plasma glucose, the total rate of appearance of UDP-glucose consist of 3 contribution: gluconeogenic flux from G6P, glycogenolysis and the flux of plasma glucose into the UDP-glucose pool. This flux of glycogen into UDP-glucose is a measure of glycogen/glucose-1-phosphate (G1P) cycling (18).

Individual fluxes through enzymes. The individual fluxes through the enzymes GK, G6Pase, GS and GP were calculated according to:

\[ \text{GK} = c(glc) \times \text{totalRa(UDPglc)} + r(glc), \]

in which 2 contributions to the total flux through GK are considered, i.e. the flux of plasma glucose into UDP-glucose and glucose/G6P cycling,
G6Pase = GNG(glc) + GLY(glc),  
[15]

GS = GNG(UDPglc) + GLY(UDPglc)  
[16]

and

GP = GLY(glc) + GLY(UDPglc),  
[17]

in which 2 contributions to the total flux through GP are considered, i.e. glycogenolysis resulting in plasma glucose appearance and glycogen/G1P cycling.

**Statistics.** All values are expressed, as mean ± S.D. Statistical significance was determined using Students-t test. P<0.05 was considered as significant.

**Table 1. Effects of S4048 on the production of glucose and lactate hepatocytes and on the intracellular content of glucose-6-phosphate and glycogen in hepatocytes.** Hepatocytes were incubated for 60 minutes in Krebs-Henseleit buffer with either 10mM dihydroxyacetone or 20 mM glucose with or without S4048 (10 µM) as described in "Experimental Procedures". Glucose, lactate, glucose-6-phosphate, and glycogen were determined at the end of the incubation period by standard enzymatic procedures as described in "Experimental Procedures". S4048 was dissolved in DMSO (final concentration in the incubations, 0.5% w/v). the controls contained DMSO only.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>N</th>
<th>Product (µmole g dry wt.⁻¹)</th>
<th>Control</th>
<th>S4048</th>
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<tr>
<td><strong>Medium</strong></td>
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<td></td>
<td></td>
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<tr>
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<td>3</td>
<td>Glucose</td>
<td>381 ± 17</td>
<td>22 ± 9ᵃ</td>
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<tr>
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<td>3</td>
<td>Lactate</td>
<td>139 ± 26</td>
<td>277 ± 6ᵃ</td>
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<td>G-6-P</td>
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<td>1.55 ± 0.05ᵃ</td>
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<tr>
<td></td>
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<td>Glycogen</td>
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<tr>
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<td>-</td>
<td>-</td>
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<tr>
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<td>Glycogen</td>
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ᵃ = significantly different (p<0.001) between control and S4048
RESULTS

**S4048 stimulates glycogenesis and glycolysis in isolated hepatocytes.** Table 1 summarises the effects of S4048 on DHA and glucose metabolism in freshly isolated rat hepatocytes. S4048 at 10 µM completely inhibited glucose production from DHA which was accompanied by an increase in lactate production and glycogen synthesis. In the presence of glucose, S4048 caused a significantly increased lactate production and strongly induced glycogen synthesis. Cellular G6P concentrations were substantially increased in the presence of S4048, with either glucose or DHA as the substrate.

**S4048 affects plasma and hepatic parameters of glucose metabolism in conscious rats.** At the start of the experiment, plasma concentrations of glucose and insulin were similar in control and S4048-treated rats (Table 2). Plasma glucose concentration slightly increased during the experiment, i.e., by 23%, in control animals. In the animals treated with S4048, plasma glucose concentration dropped from ~4.4 to ~3.5 mM (-20%) during the first three hours of the experiment and remained unchanged thereafter. Insulin concentrations in S4048-treated rats decreased significantly by 56%, in contrast to the control group in which plasma insulin was slightly elevated (+32%). The G6P content of the liver was significantly higher at the end of the experiment in animals treated with S4048 compared to the control group (+346%) and S4048-treated animals showed an almost 13-fold increase in hepatic glycogen content. At the end of the experiment liver weight was slightly increased in S4048-treated rats (8.5±0.4 g wet wt vs 9.3±0.4 g wet wt, control vs S4048-treated, resp).

Table 2. Effects of S4048 treatment in fasted rats on plasma glucose and plasma insulin concentration and on hepatic glucose-6-phosphate and glycogen content. Rats were infused for 8 hours with or without S4048 as described in detail in "Experimental Procedures". Measurements were done prior to infusion and at time points 6, 7 and 8 h after start of infusion. Steady state measurements were performed between 6 to 8 h of infusion. Hepatic samples were taken at the end of the experiment after the animals were sacrificed.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>S4048</th>
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<tbody>
<tr>
<td><strong>Plasma glucose (mM)</strong></td>
<td>Initial 4.7 ± 0.4</td>
<td>4.4 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Steady state 5.8 ± 0.0</td>
<td>3.5 ± 0.9*</td>
</tr>
<tr>
<td><strong>Plasma insulin (ng ml⁻¹)</strong></td>
<td>Initial 0.7 ± 0.2</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Steady state 0.9 ± 0.1</td>
<td>0.2 ± 0.0*</td>
</tr>
<tr>
<td><strong>Hepatic G6P (µmole g wet wt⁻¹)</strong></td>
<td>End 0.5 ± 0.1</td>
<td>2.7 ± 0.3*</td>
</tr>
<tr>
<td><strong>Hepatic glycogen (µmole glc g wet wt⁻¹)</strong></td>
<td>End 17.8 ± 7.2</td>
<td>225.0 ± 41.1*</td>
</tr>
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</table>

* Significantly different (p<0.05) between control and S4048
* Significantly different (p<0.05) between initial and steady state within the groups

**S4048 induces massive periportal glycogen accumulation in the liver.** Figure 2A confirms that glycogen was almost absent in the livers from control rats. In livers of S4048-treated rats (Figure 2B), on the other hand, massive amounts of PAS-positive material were present, indicating a high content of glycogen: most of the glycogen was present in periportal hepatocytes, *i.e.*, the cells surrounding the portal vein.
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Figure 2. Effect of S4048 on glycogen accumulation and distribution in the liver. Livers of rats infused with either vehicle or S4048 for 8 h were treated with PAS to stain for glycogen and were examined by light microscopy. In (A) a representative micrograph is shown of a liver of vehicle-treated rat and in (B) for a S4048-treated rat. PV indicates perivenous area and PP indicates periportal area.

S4048 changes partitioning of G6P without altering gluconeogenic flux to G6P. Figure 3 shows the effects of S4048 treatment on total glucose production (Figure 3A) and on total UDP-glucose production (Figure 3B). The total glucose production rate decreased from 39.9±3.8 µmole kg⁻¹ min⁻¹ in the control animals to 19.6±4.2 µmole kg⁻¹ min⁻¹ in animals treated with S4048. At the same time, the total UDP-glucose production significantly increased from 19.8±1.8 in the control animals to 30.7±1.5 µmole kg⁻¹ min⁻¹ in S4048-treated rats. Compared to control animals, the total gluconeogenic flux into G6P was not changed significantly in animals treated with S4048 (Figure 4; 33.3±2.0 vs 33.2±2.9 µmole kg⁻¹ min⁻¹ in control vs. S4048-treated, respectively). The flux of de novo synthesised G6P directed to plasma glucose, however, was significantly decreased in S4048-treated animals as compared to controls (from 20.8±1.7 to 11.6±2.4 µmole kg⁻¹ min⁻¹). In contrast, the flux of newly synthesised G6P directed to UDP-glucose significantly increased in S4048-treated animals as compared to controls (from 12.5±0.4 to 21.6±0.8 µmole kg⁻¹ min⁻¹). As a consequence, the partitioning of newly synthesised G6P changed from 62±1 % into plasma glucose and 38±1 % into glycogen in control rats to 35±5 % into plasma glucose and 65±5 % into glycogen in S4048-treated rats.

Figure 3. Effects of S4048 treatment in fasted rats on total plasma glucose production (A) and UDP-glucose production (B). The metabolic fluxes were calculated using the equations for totalRa(glc) (IV; Figure 1) and totalRa(UDPglc) (VI; Figure 1) in (A) and (B), respectively, as described in "Experimental Procedures". *Significantly different between control and S4048.
**Figure 4.** Effect of S4048 inhibitor on gluconeogenesis flux and partitioning. The gluconeogenic fluxes are shown directed into the plasma glucose pool (dark grey bar) and into the UDP-glucose pool (light grey bar). The fluxes were calculated using the equations for GNG(glc) (I + IV; Figure 1) and GNG(UDPglc) (I + VI; Figure 1), respectively, as described in “Experimental Procedures”. * Significantly different between control and S4048.

**S4048 affects in vivo fluxes through enzymes involved in G6P metabolism.** In Figure 5 the values of the various fluxes through enzymes involved in G6P metabolism are shown, as far as these flux rates could be estimated by the isotopic model used. Administration of S4048 resulted in a decrease of the flux through G6Pase from 39.9±3.8 µmole kg⁻¹ min⁻¹ to 19.6±4.2 µmole kg⁻¹ min⁻¹ and through GK from 10.1±0.4 µmole kg⁻¹ min⁻¹ to 1.6±0.5 µmole kg⁻¹ min⁻¹. Glucose/G6P cycling decreased from 6.4±0.1 µmole kg⁻¹ min⁻¹ to 0.6±0.3 µmole kg⁻¹ min⁻¹. The flux through GS increased upon administration of S4048 from 19.8±1.8 µmole kg⁻¹ min⁻¹ to 30.7±1.5 µmole kg⁻¹ min⁻¹, whereas the flux through GP was almost unaffected (16.9±5.3 vs 15.6±2.0 µmole kg⁻¹ min⁻¹). Glycogen/G1P cycling increased from 1.7±2.1 to 7.8±0.6 µmole kg⁻¹ min⁻¹.

**Figure 5.** Effects of S4048 treatment in fasted rats on the fluxes through hepatic carbohydrate pathways. The metabolic fluxes in vehicle-treated rats are shown in light grey bars whereas the metabolic fluxes in S4048-treated rats are shown in dark grey bars. Individual fluxes were calculated as described in "Experimental Procedures", using the equations for GK (Figure 1, III), G6Pase (Figure 1, IV), GS (Figure 1, VI) and GP (Figure 1, II). Glucose/G6P and glycogen/G1P recycling were calculated using the equations for r(glc) and
S4048 treatment induces rapid changes in gene expression. Expression of genes involved in hepatic carbohydrate metabolism were studied by semi-quantitative PCR (Figure 6). Treatment with S4048 resulted in markedly increased mRNA levels of the genes encoding GLUT-2, G6PH and G6PT, GS and L-PK within the 8h time-frame of the experiment. In contrast, GK gene expression were strongly suppressed. As expected on the basis of flux measurements, the mRNA levels of PEPCK and GP were unaffected.

**DISCUSSION**

This study reveals striking, rapid effects of acute pharmacologic inhibition of G6PT by S4048 on hepatic glucose metabolism in fasted rats. Absence of G6PT activity underlies glycogen storage disease type Ib. In the clinical presentation of this inborn error of metabolism both the primary metabolic effects, due to the absence of the translocase activity, and the metabolic...
adaptations that occur contribute to the characteristic phenotype observed in these patients, i.e., fasting induced hypoglycemia, hyperlactacidemia as well as hyperlipidemia. A similar combination of primary and secondary effects is present in the recently generated G6PH knock-out mice (22).

In view of the pivotal role of G6P in glucose metabolism, we interpret the changes in hepatic glucose metabolism induced by S4048 as a coordinate response aimed at maintenance of hepatocellular G6P concentration. Several experimental studies (23-25) and theoretical considerations (26), have emphasized the importance of maintaining constant concentrations of intermediates, that are shared by several metabolic pathways. For G6P metabolism in muscle, Shulman and coworkers (23,24) proposed that changes in GS activity did not control glycogen synthesis but, instead, were aimed at maintaining a constant intracellular G6P concentration. Aiston et al. (25) proposed that activity of G6Pase in hepatocytes changed in such a way that hepatocellular G6P concentration was maintained during adenoviral G6Pase overexpression in freshly isolated hepatocytes. In line with this proposal it was shown previously that inhibition of G6PT in rats resulted in an increase in steady state mRNA levels of G6PH (27). From a theoretical point of view, Kacser and Acerenza (26) argued that homeostasis of shared intermediates is necessary for independent regulation of metabolic pathways involved.

The validity of the isotopic model and the MIDA approach has been substantiated in various studies, although still some controversy remains (28-40). Like any method, the MIDA approach is based on certain assumptions. Several of these assumptions have been addressed both experimentally (36, 39-42) and theoretically (43) and the outcome of these studies have been critically reviewed (43,44). The methodology tolerates a wide range of label disequilibrium in triose-phosphate pool. It may be sensitive to isotope gradients in the triose-phosphate pool across the liver, i.e., those in perportal and perivenous cells, but the existence of such a gradient has not yet been proven experimentally. On the contrary, recent data by Siler et al. (33) make the existence of such a gradient unlikely. Although the applied [2-13C]-glycerol infusion rates are high in comparison with the usual infusion rates in in vivo tracer experiments, only minor confounding effects are to be expected due to [2-13C]-glycerol. Previs et al. (40) have shown in 30-h fasted mice that steady state concentrations of glycerol in plasma started to increase at a glycerol infusion rate of 60 µmole kg\(^{-1}\) min\(^{-1}\) and that the endogenous glucose production started to increase at 120 µmole kg\(^{-1}\) min\(^{-1}\). In our experiments in rats, fasted for 24 h, a [2-13C]-glycerol infusion rate of less than 10 µmole kg\(^{-1}\) min\(^{-1}\) was used. The calculated isotope mole percent enrichment of the "true triose-phosphate" precursor pool for de novo G6P synthesis (p-value) was about 15% in our experiments, indicating that the [2-13C]-glycerol infusion contributed only moderately to the total production rate of intracellular triose-phosphate. Finally direct comparison of independent isotopic methods to estimate gluconeogenesis have yielded either very similar, or slightly lower values for the MIDA method (41,45). For our comparative study these concerns are of lesser importance. We studied changes in de novo synthesis of G6P and partitioning of newly synthesized G6P brought about by acute inhibition of hepatic glucose production. Measurements were done under very similar conditions and, as a consequence, the results obtained reflect actual changes in G6P metabolism.

Quantitatively, the changes in the calculated fluxes through GS and GP brought about by S4048 were almost equal to the measured amount of glycogen found in livers of S4048 treated rats at the end of the experiment. Glycogen accumulation is the net result of the opposing fluxes through GS and GP. In the presence of S4048 the difference between the flux through GS (~30 µmole kg\(^{-1}\) min\(^{-1}\)) and GP (~15 µmole kg\(^{-1}\) min\(^{-1}\)) equals ~15 µmole kg\(^{-1}\) min\(^{-1}\). At the end of the experiment this results in 7200 µmole kg\(^{-1}\) or ~225 µmole g wet wt\(^{-1}\) of glycogen (liver weight was ~34 g wet wt kg\(^{-1}\)) matching the measured amount of glycogen
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formed (~225 µmole g wet wt⁻¹; Table 2). The increased net glycogen synthesis (~15 µmole kg⁻¹ min⁻¹) was, however, less than the decrease in endogenous glucose production (~20 µmole kg⁻¹ min⁻¹) brought about by S4048. The remainder of the decrease in total glucose production (~5 µmole kg⁻¹ min⁻¹) can be accounted for by the decrease in glucose/G6P cycling (cf. eq. 7), which decreased from ~6 µmole kg⁻¹ min⁻¹ to ~1 µmole kg⁻¹ min⁻¹ in the presence of S4048.

The de novo synthesis of G6P was unaffected by inhibition of G6PT. When gluconeogenesis would have been calculated based on the fractional contribution to plasma glucose alone, our results would have led us to conclude that gluconeogenesis was inhibited in parallel with inhibition of glucose production. By analyzing both plasma glucose and urinary p-GlcUA, however, we were able to show that the decrease in hepatic glucose production was not associated with a decrease in the gluconeogenic flux to G6P, but to a predominant partitioning of newly synthesized G6P into glycogen. Thus, no feedback inhibition on the gluconeogenic flux by its product G6P was observed in the 8 hr time-frame of the experiment. Inhibition of G6PT decreased plasma glucose and insulin concentrations as well. The rate of de novo synthesis of G6P was also not increased in the face of decreased plasma glucose and insulin concentration. Gene expression of PEPCK was found to be unaffected, in parallel with the unaffected gluconeogenic flux to G6P. The role of PEPCK in controlling the gluconeogenic flux is a matter of controversy. Although PEP-CK has been claimed to be rate-limiting in gluconeogenesis (46), measurements until now did not substantiate this claim. In hepatocytes from fasted rats, PEPCK exerted only minor control over gluconeogenesis from lactate (47). Recent data by the group of Magnuson (48), using an allelogenic CreloxP gene targeting strategy to inactivate PEPCK specifically in mouse liver, substantiate these observations. Hepatic glucose production did not diminish until activity of PEPCK in liver reached levels below 90-95% of its initial activity. Hormone-stimulated PEPCK gene expression can be repressed by high extracellular glucose concentrations in vitro after intracellular metabolism of glucose (49,50). We did not observe such a regulation. In our in vivo experiments the high intracellular G6P concentration was, however, accompanied by low plasma glucose and insulin concentrations, which might contribute to the observed difference in outcome of the in vitro findings and our study (49,50).

Acute inhibition of G6PT in vivo raised hepatocellular G6P concentration and abolished glucose/G6P cycling. The simultaneous action of G6PH, G6PT and GK represents a homeostatic mechanism aimed at maintaining a constant intracellular G6P concentration (25). GK enzyme activity does not experience feedback inhibition by G6P (see ref 51 and refs therein), so that G6P in excess of metabolic demands must be hydrolyzed by G6PH. Inhibition of G6PT interferes with this homeostatic mechanism. In isolated hepatocytes, inhibition of G6PT also increased glucose incorporation into glycogen and glycogenolysis. This emphasizes the importance of glucose/G6P cycling in hepatocellular glucose metabolism. As has been reported previously, high intracellular concentrations of G6P markedly stimulated expression of the gene encoding G6PH (27). This was confirmed in the present study. GK gene expression, on the other hand, is strongly reduced by S4048 treatment. This suggest that at high intracellular G6P concentrations a negative control system is operational to down-regulate GK expression, quite different from in vitro studies on GK gene expression (3,4). In the latter studies, GK gene expression was found not to depend on intracellular glucose metabolism. Irrespective of the very low GK mRNA levels some glucose phosphorylation did still occur, as is evident from our calculations. It is important to realize that 1/2 of the GK protein is relatively long (30 h, cf. ref. 51) in comparison with the duration of the experiment. The role of increased expression of the gene encoding for GLUT-2 in maintaining a constant hepatocellular G6P concentration is not clear, particularly since very recent data show that glucose production from pyruvate is not affected in hepatocytes isolated
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from GLUT2 knock-out mice (52). The absence of GLUT2 did, however, lead to a sustained elevated intracellular G6P. This indicates a role of GLUT2 in regulating intracellular G6P concentration by exporting cytosolic glucose, thereby preventing rephosphorylation of glucose by GK.

Glycogen synthesis was strongly stimulated upon inhibition of G6PT. This was accompanied by an increased glycogen/G1P cycling. Apparently both GS and GP were simultaneously active. G6P is an allosteric activator of GS \( b \) in hepatocytes and also activates GS phosphatase, while glucose is a competitive inhibitor of GP \( a \) activity and promotes the dephosphorylation and inactivation of GP (1). Inhibition of G6PT raised hepatocellular concentration of G6P. This may have stimulated GS \( b \) activity and/or promoted its dephosphorylation into its active \( a \) form by glycogen-associated protein phosphatase-1. As a consequence, flux through GS increased. On the other hand, inhibition of G6PT also decreased the concentration of plasma glucose, so that the activity of GP \( a \) may remain high.

The observations on glycogen/G1P cycling are in line with studies by others with in vivo \(^{13}\)C-MRS on the simultaneous synthesis and degradation of liver glycogen during a D-[\(^{13}\)C]-glucose infusion in fasted and fed rats (53) and humans (17). The continuous degradation and synthesis of glycogen adds to G6P homeostasis. Newsholme and Crabtree (54) have argued that, in the presence of substrate cycling, large fluctuations in concentrations of intermediates can be dampened by relatively small changes in the rates of the opposing reaction, constituting the substrate cycle. In our study GS gene expression was increased while gene expression of GP was unaffected. The physiological importance of these changes in regulation of glycogen metabolism is not yet clear, but it may point to a control loop, at the level of gene expression, by which G6P stimulates its own deposition into glycogen, which adds to the proposed homeostatic mechanism.

In freshly isolated hepatocytes, in short term incubations, glycolysis was strongly stimulated in the presence of S4048 and glucose was more effectively converted into lactate. These observations point to the importance of glucose/G6P cycling in glucose metabolism in this in vitro experimental system. Likewise, in vivo treatment of rats with S4048 resulted in an increased plasma lactate concentration and the expression of the gene encoding L-PK is markedly upregulated. Regulation of L-PK critically depends on glucose metabolism. Both, G6P and X5P have been implicated in this regulation (see 2 for review).

Results of a number of studies on glycogen synthesis are in line with the proposed notion that the gluconeogenic flux to G6P is not subjected to acute changes (cf. 55) under various experimental conditions. For instance, during refeeding after a period of fasting, glycogen is synthesized by two metabolic routes; a "direct" one (Glc-> G6P-> UDP-Glc-> glycogen; Figure 1, III + VI) and an "indirect" one (Glc-> C\(_3\)-compound-> G6P-> UDP-Glc-> glycogen; Figure 1, III + V + I + VI) (55). After glucose phosphorylation and glycolysis the "indirect" pathway is identical to the gluconeogenic flux to G6P with subsequent partitioning of newly synthesized G6P into glycogen. Partitioning of G6P will determine whether newly synthesized G6P will go to either glucose production or glycogen synthesis. This partitioning is a function of the relative activities of the enzymes involved in G6P metabolism. In case of NIDDM, with inappropriately high hepatic glucose production, this partitioning mechanism may be perturbed. In fact, it has been reported that in patients with NIDDM the activity ratio of G6Pase over GK was increased (56). Increasing the activity ratio of G6Pase over GK by adenovirus-mediated overexpression of the gene encoding G6PH was associated with increased hepatic glucose production in conscious rats (57). Overexpressing the gene encoding for the GK and thereby decreasing the activity ratio of G6Pase over GK, resulted in a decreased hepatic glucose production in conscious rats (58).

In summary, this study showed that acute pharmacologic inhibition of G6PT resulted in a marked increase in hepatocellular G6P and glycogen without affecting the gluconeogenic
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flux to G6P. The expression of genes of enzymes in glucose cycling, glycogen synthesis and glycolysis was changed in such a way to maximize the ability to deposit newly synthesized G6P into glycogen in order to maintain cellular G6P homeostasis.

FOOTNOTES

1 The abbreviations used are: GK, glucokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.2), G-6-Pase, glucose-6-phosphatase activity; G6PH, glucose-6-phosphatase hydrolase (D-glucose-6-phosphate phosphohydrolase EC 3.1.3.9); G6PT, glucose-6-phosphatase translocase; GLUT-2, glucose transporter type 2; GP, glycogen phosphorylase (glycogen 1,4-α-D-glucan:orthophosphate α-D-glucosyltransferase, EC 2.4.1.1); GS, glycogen synthase (UDPglucose:glycogen 4-α-D-glucosyltransferase, EC 2.4.1.11); PEP-CK, phosphoenolpyruvate carboxykinase (GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32), L-PK, liver type pyruvate kinase (ATP:pyruvate O2-phosphotransferase, EC 2.7.1.40); DHA, dihydroxyacetone; DMSO, dimethylsulfoxide; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; G1Pase, glucuronide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; TMS, trimethylsilyl; X5P, xylulose-5-phosphate.

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