Acute Inhibition of Glucose-6-Phosphate Translocator Activity Leads to Increased De Novo Lipogenesis and Development of Hepatic Steatosis Without Affecting VLDL Production in Rats

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Glucose-6-phosphatase (G6Pase) is a key enzyme in hepatic glucose metabolism. Altered G6Pase activity in glycogen storage disease and diabetic states is associated with disturbances in lipid metabolism. We studied the effects of acute inhibition of G6Pase activity on hepatic lipid metabolism in nonanesthetized rats. Rats were infused with an inhibitor of the glucose-6-phosphate (G6P) translocator (S4048, 30 mg·kg⁻¹·h⁻¹) for 8 h. Simultaneously, [1-¹³C]acetate was administered for determination of de novo lipogenesis and fractional cholesterol synthesis rates by mass isotopomer distribution analysis. In a separate group of rats, Triton WR 1339 was injected for determination of hepatic VLDL-triglyceride production. S4048 infusion significantly decreased plasma glucose (−11%) and insulin (−48%) levels and increased hepatic G6P (201%) and glycogen (182%) contents. Hepatic triglyceride contents increased from 5.8 ± 1.4 μmol/g liver in controls to 20.6 ± 5.5 μmol/g liver in S4048-treated animals. De novo lipogenesis was increased >10-fold in S4048-treated rats, without changes in cholesterol synthesis rates. Hepatic mRNA levels of acetyl-CoA carboxylase and fatty acid synthase were markedly induced. Plasma triglyceride levels increased fourfold, but no differences in plasma cholesterol levels were seen. Surprisingly, hepatic VLDL-triglyceride secretion was not increased in S4048-treated rats. These studies demonstrate that inhibition of the G6Pase system leads to acute stimulation of fat synthesis and development of hepatic steatosis, without affecting hepatic cholesterol synthesis and VLDL secretion. The results emphasize the strong interactions that exist between hepatic carbohydrate and fat metabolism. Diabetes 50:2591–2597, 2001

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hosphorylation and dephosphorylation of glucose by glucokinase and glucose-6-phosphatase (G6Pase), respectively, are key steps in hepatic glucose uptake and release. The balance between the activities of these enzymes represents an important site for the control of hepatic glucose production (1,2). G6Pase is located in the endoplasmic reticulum (ER) of liver, kidney, and, as recently shown, intestinal cells (3). The glucose-6-phosphate (G6P) metabolizing machinery consists of a putative translocator (4,5) that transports G6P from the cytosol into the ER lumen and a catalytic subunit that converts G6P to glucose and inorganic phosphate (6). The catalytic subunit is localized to the inner ER membrane. Interestingly, there are several indications to suggest that this site of regulation of glucose metabolism is linked to that of hepatic lipid metabolism. G6Pase activity is increased in patients and animal models of diabetes (2,7,8), probably contributing to increased hepatic glucose production in these conditions. Diabetes is generally associated with hyperlipidemia, which has been found to be mainly due to overproduction of VLDL-triglycerides in type 2 diabetes (9–11). Deficiency of G6Pase activity, the metabolic basis of glycogen storage disease type I (GSD-1), also leads to abnormalities in lipid metabolism, characterized by severe hypertriglyceridemia and hypercholesterolemia (12–15). Glycogen storage disease (GSD) is caused by mutations in the genes encoding either the putative translocator (type non-1a) (4,5) or the catalytic subunit (type 1a) (6,16,17) of the G6Pase system. Overexpression of hepatic glucokinase also leads to hyperlipidemia in fed rats (18). Brown et al. (19) showed that the phosphorylation process is important for regulation of assembly and secretion of triglyceride-containing VLDLs by hepatocytes. Little is known about the mechanisms underlying the apparent paradox that hyperlipidemia de-
for isolation of VLDL/LDL particles, and the animals were killed. Lipoproteins were isolated according to Pietzsch et al. (25) using a solution of 15.3% NaCl and 35.4% KBr in saline with a density <1.019 g/ml. Plasma (0.5 ml) was overlayed with 0.6 ml of the NaCl–KBr solution, centrifuged for 100 min at 120,000 rpm and 4°C in a Beckman Optima TLX Ultracentrifuge (Beckman Instruments, Palo Alto, CA), and the VLDL fraction was collected by tube slicing and was frozen until analysis. VLDL particle size was calculated as described by Beil et al. (26).

Analytical procedures. Hepatic lipids were extracted using the method of Bligh and Dyer (27). To determine plasma and hepatic triglyceride and cholesterol concentrations, assay kits were obtained from Hoffmann-La Roche (Basel). To determine plasma and hepatic phospholipid and plasma free fatty acid (FFA) concentrations, assay kits were obtained from Waco Chemical (Marburg, Germany). Plasma β-hydroxybutyrate was measured using a commercially available kit from Sigma. Total protein content of tissue homogenates was determined according to Lowry et al. (28). Plasma insulin was determined by radioimmunoassay (RI-13K; Linco Research, St. Charles, MO).

Plasma glucose concentration was determined enzymatically by use of the Beckman glucose analyzer II. After extraction with a 1:1 mol/l KOH solution, hepatic glycogen was determined by sonication. The extract was incubated for 30 min at 90°C, and then brought to pH 4.5 by adding 3 mol/l acetic acid. Precipitated protein was removed by rapid centrifugation (10,000 rpm for 1 min). Glycogen was converted to glucose by treating the samples with amyloglucosidase, followed by assay of glucose at pH 7.4 with hexokinase, and G6P dehydrogenase (29). For the determination of G6P, liver samples were treated by sonification in a 5% (wt/vol) HClO4 solution. Precipitated protein was removed by rapid centrifugation at 10,000 rpm for 1 min in a cold microcentrifuge, and the supernatant was neutralized to pH 7.0 by adding small amounts of a mixture of 2 mol/l KOH and 0.3 mol/l MOPS. G6P was determined fluorometrically with NADP+ and G6P dehydrogenase (30).

Activities of liver enzymes, i.e., alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by routine chemical chemistry procedures.

Liver histology. To visualize fat deposition in the liver, staining with Oil-red-O was performed on 4-μm frozen liver slices, and counterstaining was performed with hematoxylin, according to standard procedures.

Gas chromatography/mass spectrometry analysis. For gas chromatography/mass spectrometry analysis, plasma cholesterol was extracted and derivatized as described elsewhere (31). Palmitate from isolated VLDL fractions was trans-methylated according to Lepage et al. (32).

Cholesterol and fatty acid derivatives were analyzed on a magnetic sector mass spectrometer (70–2500; Micromass, Manchester, U.K.) using a CP-Sil 19 column (Chrompack, Middelburg, the Netherlands) for assessment of isotope distribution patterns. For cholesterol samples, the oven temperature was increased from 280°C to 300°C in the course of a [1-13C]acetate infusion to be determined by analyzing the isotopomer pattern of the molecules of interest. This isotopomer pattern is developed and eventually will be aimed at treating hyperglycemic conditions in type 2 diabetes (20–22). Infusion of members of this class of compounds in anesthetized rats resulted in reduction of blood glucose levels and increased concentrations of intrahepatic G6P and glycogen (21,22). Recently, it has been reported that acute inhibition of G6Pase activity also increases hepatic triglyceride concentrations (21). As a result of their mode of action, chlorogenic acid derivatives induce a situation resembling GSD-1 and provide excellent tools to unravel the interactions between carbohydrate and lipid metabolism.

In the present study, we acutely inhibited G6Pase activity by infusion of the chlorogenic acid derivative S4048 in vivo in conscious, unrestrained, nondiabetic rats. We questioned whether acute increases in hepatic G6P concentrations would lead to increased hepatic de novo lipogenesis, cholesterogenesis, and VLDL-triglyceride secretion.

**RESEARCH DESIGN AND METHODS**

**Animals.** Male Wistar rats (Harlan Laboratories, Zeist, the Netherlands), weighing between 290 and 350 g (means ± SD: 318 ± 25 g), were used to study the in vivo effects of S4048. To allow infusion and blood collection in freely moving, unrestrained animals, rats were equipped with two permanent heart catheters via the right jugular vein as described by Kuipers et al. (23). After surgery, animals recovered for a period of 7 days in individual cages in a temperature-controlled room (20°C) with food and water available ad libitum. At 8 h before the start of the experiment, the cages were cleaned and the food was removed.

**Experimental procedures.** Animals received an intravenous infusion of the G6P translocator inhibitor (S4048, 30 mg·kg⁻¹·h⁻¹, infusion rate 3 ml/h) or of the solvent (phosphate-buffered saline [PBS] with DMSO) for 8 h. Rats were allowed to move freely throughout the experiment, and the animals did not show signs of stress. The S4048 compound was synthesized by the chemical department of Aventis Pharma (Frankfurt, Germany) (21). At the infusion rate of 3 ml/h, animals were anesthetized with sodium-pentobarbital, and a temperature-controlled room (20°C) with food and water available ad libitum. After the surgical procedures and recovery as described above, the rats were infused with S4048 or its solvent and, after 3 h of infusion, received an intravenous Triton WR 1339 injection (Tyloxapol; Sigma Chemical, St. Louis, MO) as a (12% wt/wt) solution dissolved in saline in a dose of 5 ml/kg body wt. Triton WR 1339 was dissolved in 20% DMSO in PBS (vol/vol), and PBS was added to reach a final concentration of 6.1% DMSO (vol/vol). The solution was then immediately adjusted to pH 7.4. Before the infusion, a basal blood sample (~300 μl) was taken to determine the baseline values of the metabolites studied. During the infusion, blood samples (~200 μl) were taken after 30, 60, 120, 240, 360, and 420, and 480 min. Samples were heparinized and immediately placed on ice and centrifuged at 5,000 rpm for 10 min at 4°C. The plasma was stored at −20°C until analysis. Despite the relatively high infusion rate, hematocrit levels did not fall significantly during the experiment. At the end of the infusion period, animals were anesthetized with sodium-pentobarbital, and a large blood sample was taken by heart puncture. The abdomen was opened, and the liver was rapidly exposed, excised, and stored in parts at −80°C for measurement of G6P and glycogen content, lipid analysis, and RNA isolation or rapidly frozen in liquid isopentane for histological analysis (see below).

**In vivo VLDL-triglyceride production.** The effects of S4048 on hepatic VLDL production were studied in a separate experiment. After the surgical procedures and recovery as described above, the rats were infused with S4048 or its solvent and, after 3 h of infusion, received an intravenous Triton WR 1339 injection (Tyloxapol; Sigma Chemical, St. Louis, MO) as a (12% wt/wt) solution dissolved in saline in a dose of 5 ml/kg body wt. Triton WR 1339 blocks lipolysis of lipoproteins, which accumulate over time in plasma, allowing for the calculation of hepatic VLDL-triglyceride production rates (24). To exclude any interference of the solvent containing DMSO on VLDL-triglyceride secretion, a separate group of rats received a saline infusion. After Triton WR 1339 was injected, blood samples were taken after 30, 60, 120, and 180 min for measurement of triglyceride concentrations. VLDL production rates were calculated from the slope of the linear triglyceride accumulation curves in time. After 180 min, a large blood sample was taken.
CT, and the anti-sense primer was TCG AAG GCT ACA CAA GCT CCA AAA GAA TA (34); for sterol regulatory element binding protein (SREBP)-1 (SREBP-1a and -1c, GenBank L16995), the sense primer was CCT GTG TGT ACT GGT CCT CTG TGT TAC T; for SREBP-2 (GenBank U02031), the sense primer was CAA TGG CAC GCT GAC CCT TG, and the anti-sense primer was ATG GCC TTC CTC AGA TGG CCA; for HMG-CoA reductase (GenBank M29249), the sense primer was GAC ACT TAC AAT CTG TAT GAT G, and the anti-sense primer was CTT GGA GAG GTA AAA CTG CCA; for HMG-CoA synthase (GenBank X52625), the sense primer was TAC GAT GGT GTA GAT GCT GG, and the anti-sense primer was AGT TCT TCT GTG CTT TTC ATC CAC; for apolipoprotein B (apoB) (GenBank M14952), the sense primer was GAC ATG GTG AAT GGA ATC ATG, and the anti-sense primer was TGA AGA CTC CAG ATG AGG AC (34); for /H9252-actin (GenBank M12481), the sense primer was AAC ACC CCA GCC ATG TAC G, and the anti-sense primer was ATG TCA CGC ACG ATT TCC C; for microsomal triglyceride transfer protein (MTP) (GenBank LA7970), the sense primer was ATC TGA TGT GGA CGT TGT GT, and the anti-sense primer was CCT CTA TGC GGA GGA GCC ATG TAG TG; for carnitine palmitoyltransferase I (CPT-I) (GenBank L07736), the sense primer was GCA TCA TCA CTG GTG TGT TC, and the anti-sense primer was TCT CCA TGG CGT AGT AGT TG. For each primer set, an increasing number of PCR cycles was performed while other conditions were fixed in order to determine the optimal number of cycles, which was chosen as the number halfway through the exponential phase. The PCR products were separated on 2.5% agarose gels. Images were made using a CCD video camera (Image Master VDS system; Pharmacia, Upsalla, Sweden).

Statistical analysis. All values reported are means ± SD. Significance was determined using the nonparametric Mann-Whitney U test for unpaired data. Differences were considered significant at P < 0.05.

RESULTS
Effects of S4048 on plasma parameters. Figure 1 shows the effects of S4048 infusion on plasma glucose, insulin, cholesterol, triglyceride, and FFA concentrations. Infusion of S4048 modestly decreased plasma glucose concentrations (P < 0.05), especially during the first 2 h of the experiment, with a subsequent significant decrease (P < 0.05) in plasma insulin concentrations. Both plasma glucose and insulin concentrations reached values approaching those in control rats at the end of the experiment. Triglyceride concentrations increased significantly during the course of the experiment from 0.4 ± 0.1 to 1.9 ± 0.6 mmol/l in S4048-treated rats. Cholesterol levels did not change during the course of the experiment, whereas FFA levels displayed a modest increase during S4048 infusion. The ketone body /H9252-hydroxybutyrate concentration was 0.54 ± 0.23 mmol/l in control and 0.75 ± 0.71 mmol/l in S4048-treated animals. No effects of DMSO, S4048, or Triton WR1339 administration on ASAT or ALAT activities in plasma were found, indicating an absence of direct hepatotoxic actions of these compounds. A modest increase was found in ASAT and ALAT activities in the animals receiving all three compounds simultaneously.

Effect of S4048 on hepatic parameters. Infusion of S4048 clearly affected hepatic carbohydrate and lipid contents (Table 1). Liver weight, expressed as percentage of body weight, was 3.0 ± 0.4 and 3.3 ± 0.2% (NS) in the control and S4048 groups, respectively. Hepatic G6P and glycogen contents both increased almost threefold after S4048 infusion. Total cholesterol content was not affected, although a higher relative cholesteryl ester content was found. Triglyceride content was markedly increased in the S4048 group, i.e., S4048 induced massive steatosis within the 8-h time frame of the experiment.

Figure 2 shows representative sections of livers from solvent- and S4048-treated rats, stained for neutral fat by Oil-red-O. Massive amounts of neutral fat were present in livers of S4048-treated rats, mainly in peribiliary areas of the hepatic lobuli. In contrast, the relatively small amounts of neutral fat present in the control liver were concentrated around the central veins, i.e., in perivenous areas of the lobuli.
Effect of S4048 on de novo lipogenesis and choles-
terogenesis. In Table 2, the effects of S4048 on palmitate
and cholesterol synthesis rates are summarized. Plasma
fractional cholesterol synthesis rates were similar in both
groups of rats. Fractional de novo lipogenesis in plasma
VLDL and liver were increased almost threefold in the
treated animals. When the increased hepatic palmitate
content was taken into account, the absolute amount of
newly synthesized palmitate was increased
10-fold in the
S4048-treated group compared with the control group.
Calculated enrichments of the acetyl-CoA pools showed
significantly decreased values in the S4048-treated rats.

S4048 induces expression of lipogenic genes. Figure 3
shows the mRNA levels of ACC, FAS, HMG-CoA reduc-
tase, HMG-CoA synthase, and SREBP-1 and -2 in control
and S4048-treated rats, as determined by a semiquantita-
tive reverse transcriptase–PCR approach. Intensities of
bands were normalized to those of β-actin (Fig. 3B). The

![FIG. 2. Oil-red-O-stained liver sections of a control rat (A) and an
S4048-treated rat (B). S4048 treatment results in increased fat depo-
sition in the liver, with a preferential localization in the perportal area
of the liver lobulus. PP, perportal area, surrounding the portal triad;
PV, perivenous area, surrounding the hepatic vein.](image)

![FIG. 3. Image of PCR products of genes upregulated by S4048, i.e., ACC
and FAS, compared with β-actin (A). The mRNA levels, which were
determined by reverse transcriptase–PCR (n = 3 in both groups) and
normalized to β-actin mRNA, are expressed as mean percent compared
with control values (B). Levels are shown for ACC, FAS, HMG-CoA
synthase (hmgs), HMG-CoA reductase (hmgr), and SREBP-1 and -2. □,
Control rats; ■, S4048-treated rats. *Significantly different from con-
trol values.](image)
Pathways of de novo lipogenesis are under transcriptional control of SREBPs (37,38), a group of transcription factors (SREBP1a, -1c, and -2) that regulate the expression of genes involved in cholesterol, fatty acid, and glucose metabolism. SREBP-1 gene knockout mice show a very low basal expression of ACC and FAS and hardly possess the ability to upregulate de novo lipogenesis (37). In contrast, overexpression of the nuclear form of SREBP-1a leads to massive steatosis and increased de novo lipogenesis, albeit in the absence of hypertriglyceridemia (39). In S4048-treated rats, SREBP-1 mRNA expression was not significantly induced, but this obviously does not exclude the possibility of direct SREBP-1-mediated activation of gene expression, particularly in view of the recent results of Foretz et al. (40). These authors have shown that enhancement of mRNA expression of ACC and FAS by SREBP-1c in isolated hepatocytes critically depends on the presence of glucose in the medium. Furthermore, it is well-established that glucose exerts stimulatory effects on lipogenic gene expression only after being metabolized to either G6P (41) or xylose-5-phosphate (42). In a recent study (18), it was shown that overexpression of glucokinase in fed rats, leading to increased G6P concentrations, resulted in a marked increase in plasma triglyceride levels. In light of the threefold increase in G6P concentration in the liver of rats infused with S4048, potentiation of transcriptional activity of SREBP-1 by G6P is highly likely to occur in our model.

Despite increased production of the obligatory precursor, i.e., acetyl-CoA, our stable isotope study showed unaffected cholesterol synthesis rates during infusion of S4048 and unaffected hepatic mRNA levels of HMG-CoA synthase and HMG-CoA reductase. In accordance, overexpression of glucokinase resulted in an increased hepatic G6P content and did not increase plasma cholesterol levels (18). SREBP-2 is a strong regulator of cholesterol synthesis, it has the ability to upregulate various genes involved in the cholesterogenic pathway, such as HMG-CoA synthase and HMG-CoA reductase. In accordance, overexpression of SREBP-2 mRNA levels were not affected in the S4048-treated animals. These combined results clearly demonstrate that in spite of the common regulatory mechanisms involved, i.e., SREBP-modulated activation of gene expression, de novo lipogenesis and cholesterogenesis are differentially regulated under conditions associated with increased glycolytic flux in rat liver.

Partial inhibition of G6Pase for an 8-h period was associated with massive steatosis. Fat accumulation was much more pronounced in hepatocytes located in the zone surrounding the portal vein and hepatic artery (periportal area) than in those surrounding the hepatic vein (perivenous area). This is probably related to the predominant peripo lar localization of G6Pase (45). Increased de novo lipogenesis contributed to the development of steatosis, but the quantitative contribution of newly synthesized fatty acids to the steatosis appeared rather limited, i.e., was <10%. Furthermore, VLDL-triglyceride secretion was similar in both groups (see below) and thus did not differentially influence hepatic lipid stores. Increased fat uptake, therefore, must have contributed, either in the form of VLDL/IDL-triglycerides or as FFAs, although plasma FFA concentrations were only moderately in-
creased on treatment. The latter, however, does not exclude an enhanced FFA flux to the liver. Furthermore, fatty acid oxidation might have been impaired in the S4048-treated rats, although similar mRNA levels of CPT-I in livers and unaffected plasma β-hydroxybutyrate concentrations were found. Malonyl-CoA is produced during the course of fatty acid synthesis and is expected to accumulate in the livers of the S4048-treated rats. Malonyl-CoA is a strong, allosterically acting inhibitor of fatty acid oxidation (46). Furthermore, the increase in hepatic triglyceride content with similar β-hydroxybutyrate concentrations strongly suggests a shift in the balance between fatty acid oxidation and esterification in the liver.

Many factors are known to influence hepatic very low density lipoprotein (VLDL)-triglyceride production and secretion. De novo lipogenesis has been suggested to be of regulatory importance for VLDL production (47). Furthermore, many studies have shown that increases in fatty acid delivery to the liver lead to increased triglyceride synthesis and are accompanied by increases in VLDL secretion (48–50). The balance between apoB synthesis and degradation is an important factor in controlling hepatic triglyceride secretion, and inhibition of protein synthesis has been shown to reduce VLDL-triglyceride secretion (51). Additionally, insulin is a well-known acute inhibitor of VLDL secretion (52–54), and insulin resistance is associated with increased VLDL-triglyceride and apoB secretion (9–11). Furthermore, recent data has shown that phosphorylation of glucose is also of importance in the regulation of assembly and secretion of triglyceride-containing VLDL (19). In our study, surprisingly, we did not find increased VLDL-triglyceride secretion in the S4048-treated rats. Moreover, neither the number nor the size of the VLDL particles were affected by S4048 infusion. In our model, the induction of de novo lipogenesis, which strongly increased hepatic triglyceride content in combination with increased hepatic G6P levels and decreased insulin concentrations, was also expected to increase the secretion of VLDL-triglycerides. However, a number of factors have to be taken into account. First of all, it is not known whether apoprotein synthesis was impaired in the S4048-treated rats. Furthermore, if glucose itself is also important in this process, lowered plasma glucose concentrations in the S4048-treated rats might cause an inability to adequately upregulate VLDL secretion. It should be stressed that in our model, G6P content is increased in the cytoplasm but probably decreased in the ER. Compartmentalization of G6P could potentially play a role in its capacity to influence VLDL secretion, but further studies are needed to clarify this phenomenon.

The observation that VLDL-triglyceride secretion was not increased in rats after S4048 treatment indicates that the hyperlipidemia observed after G6Pase inhibition must have originated from decreased triglyceride clearance. Insulin is a well-known stimulator of adipocyte lipoprotein lipase activity. In the S4048 model with a low concentration of insulin, lipoprotein lipase activity was probably decreased, leading to decreased lipolysis of VLDL-triglycerides. Indeed, studies in GSD patients have shown low lipoprotein lipase activity in GSD patients (55).

Data in the literature on the relation between G6Pase activity and lipid metabolism is confusing. Based on our results, we postulate that G6P concentrations in the liver, specifically in certain compartments, play a pivotal role in determining triglyceride concentration in liver and plasma. Altered activation of SREBP-1 and/or changes in the intrahepatic concentration of G6P in itself are most likely a better explanation than the plasma concentration of glucose or insulin for the apparently conflicting effects of G6Pase activity on hepatic lipid metabolism in diabetes and GSD.

In conclusion, acute inhibition of G6Pase activity in rats leads to increased de novo lipogenesis and massive steatosis within a relatively short time frame. Cholesterogenesis was not affected in our study, implying a dissociated regulation of cholesterol and fatty acid synthesis under the conditions used. Increased de novo lipogenesis and hepatic lipid accumulation alone is not sufficient to stimulate VLDL-triglyceride secretion. This study underlines the important function of G6P in the control of hepatic lipid metabolism.

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