

Induction of Hepatic Glucose-6-Phosphatase Gene Expression by Lipid Infusion

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The distal enzymatic step in the process of glucose output is catalyzed by the glucose-6-phosphatase (Glc-6-Pase) complex. The recently cloned catalytic unit of this complex has been shown to be regulated by insulin, dexamethasone, cAMP, and glucose. Using a combination of intralipid and/or nicotinic acid infusions and a pancreatic clamp technique, we maintained plasma free fatty acids (FFAs) at three different levels (0.26 ± 0.07 , 0.56 ± 0.09 , and 1.59 ± 0.12 mmol/l) in the presence of well-controlled hormonal and metabolic conditions. An increase in the plasma FFA concentration within the physiological range caused a rapid, greater than three-fold increase in the mRNA and protein levels of the catalytic subunit of Glc-6-Pase in the liver. These data indicate that the in vivo gene expression of Glc-6-Pase in the liver is regulated by circulating lipids independent of insulin and thus that prolonged hyperlipidemia may contribute to the increased production of glucose via increased expression of this protein. *Diabetes* 46:153–157, 1997

Glucose-6-phosphatase (Glc-6-Pase) catalyzes the final enzymatic step for hepatic gluconeogenesis and glycogenolysis (1). Glc-6-Pase is a complex of proteins with the catalytic portion deeply embedded in the endoplasmic reticulum (ER) (1). Because of its intracellular localization, it has long been suggested that the lipid composition of the ER membrane would play a pivotal role in the regulation of Glc-6-Pase activity. Indeed, some recent reports have shown an acute inhibitory effect of fatty acyl-CoA (2,3) and fatty acid ester (4,5) on Glc-6-Pase activity. In contrast, increased hepatic Glc-6-Pase activity has been reported to follow prolonged increases in dietary saturated fatty acids (6).

Important interactions between glucose and lipid metabolism have been demonstrated in skeletal muscle (7–9), liver (8–10), and β -cells (11). In particular, numerous studies have shown that the rate of endogenous glucose production is closely related to the circulating plasma FFA concentrations

(9,10,12,13) and that the majority of individuals with NIDDM have increased 24-h plasma FFA profiles (14). While the acute effects of an increase in plasma FFA availability are likely to be largely caused by its known stimulatory effect on gluconeogenesis (15), much less is known regarding the long-term consequences of increased hepatic availability of FFAs.

Indeed, dietary fatty acids have been shown to regulate the gene expression of pancreatic GLUT2 and glucokinase (16), and long-chain fatty acids can induce the expression of the genes for the adipocyte fatty acid-binding protein aP2 (17) and liver carnitine palmitoyltransferase I (CPT I) (18). Additionally, the expression of a number of hepatic lipogenic, glycolytic, and gluconeogenic genes is regulated by polyunsaturated fatty acids at the transcriptional level (19,20).

Since Glc-6-Pase mRNA and activity increase in metabolic conditions associated with high plasma FFA levels, such as fasting (21), diabetes (22–24), and high-fat feeding (6,25), we examined the effect of increasing the circulating lipid levels per se on hepatic Glc-6-Pase mRNA and protein levels in vivo. Our findings indicate that the plasma FFA concentration is an important regulator of the level of Glc-6-Pase gene expression in the liver of conscious rats.

RESEARCH DESIGN AND METHODS

Animals. Male Sprague-Dawley rats (Charles River, Wilmington, MA) were used in all studies. Rats were housed in individual cages and subjected to a standard light-dark cycle (6:00 A.M. to 6:00 P.M. and 6:00 P.M. to 6:00 A.M., respectively). From 5 to 7 days before the in vivo study, rats were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg body wt) and indwelling catheters were inserted into the right internal jugular vein and the left carotid artery. The venous catheter was extended to the level of the right atrium, and the arterial catheter was advanced to the level of the aortic arch (26–28).

Insulin/somatostatin/intralipid infusions. Studies were performed in awake, unstressed, chronically catheterized rats, using a combination of intralipid and/or nicotinic acid infusions and islet clamp techniques (8,29,30). Rats were fasted for 6 h before the in vivo studies. Briefly, a primed-continuous infusion of somatostatin ($1.2 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) and regular insulin ($1.0 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) was administered to all rats. This rate of insulin infusion was designed to increase plasma insulin modestly above basal levels and thus prevent the occurrence of hyperglycemia during intralipid infusion. When a decrease in plasma glucose levels occurred, a variable infusion of a 25% glucose solution was started and periodically adjusted to clamp the plasma glucose concentration at ~ 7 mmol/l for 5 h. Protocol 1 was designed as a time-control study (5 h), with mildly elevated plasma insulin concentrations and basal glucose and FFA levels. Protocol 2 was designed to decrease the plasma FFA concentration by infusing nicotinic acid, an inhibitor of lipolysis, at the rate of 30 mg/h after a basal period of 2 h and for a duration of 3 h. Protocol 3 was designed to increase the plasma FFA levels for 3 h by infusing a 10% triglyceride emulsion (Lyposin 10%, Abbott, North Chicago, IL) at the rate of 1.5 ml/h after a basal period of 2 h (Fig. 1). Nicotinic acid was infused at a rate identical to that used in protocol 2 in order to control for the potential effect of this drug on Glc-6-Pase gene expression. Blood samples ($\sim 25 \mu\text{l}$) were taken every 10 min to monitor the plasma glucose concentrations and adjust the rates of glucose infusion. Plasma samples for determination of plasma insulin, glucagon, and FFA concentrations were obtained every 30 min throughout the study. At the end of the in vivo studies, each rat was anesthetized (pentobarbital 60 mg/kg

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ER, endoplasmic reticulum; FFA, free fatty acid; PMSF, phenylmethylsulfonyl fluoride; SSC, sodium chloride–sodium citrate.

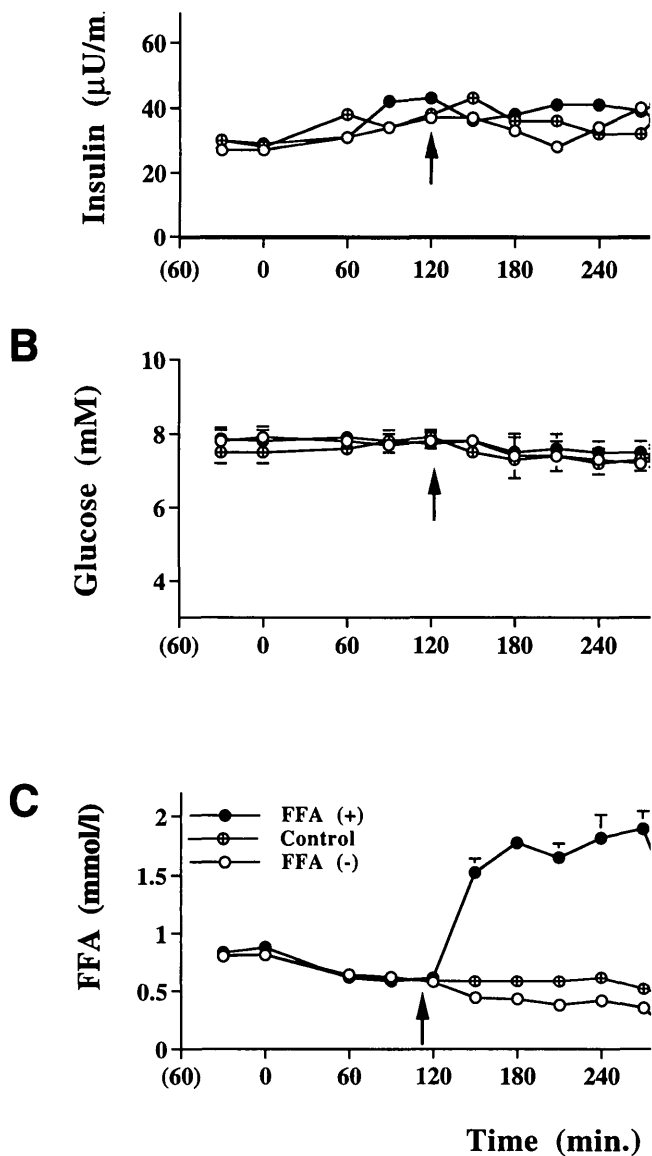


FIG. 1. Time course of the plasma insulin (A), glucose (B), and FFA (C) concentrations during the pancreatic clamp studies. To control the pancreatic hormone concentrations during the in vivo studies, somatostatin and insulin were infused during 5 h of saline (control studies) or 2 h of saline, followed by either 3 h of nicotinic acid infusion alone [FFA (-)] or nicotinic acid with lipid/heparin infusion [FFA (+)]. Insulin was infused at the rate of $1.0 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ to generate plasma hormone concentrations mildly increased above basal levels.

body wt, i.v.), the abdomen was quickly opened, portal vein blood was obtained, and the liver was freeze-clamped in situ with aluminum tongs precooled in liquid nitrogen. The time from the injection of the anesthetic until freeze-clamping of the liver was <45 s. Tissue samples were stored at -80°C until analysis. The protocol was approved by the Institutional Animal Care and Use Committees of Albert Einstein College of Medicine.

Immunoblotting analysis. Microsomes were prepared according to the method of van de Werve (31) and Rossetti and colleagues (24,32). Briefly, liver tissue (100 mg) was homogenized in 10 volumes of a Tris-sucrose-PMSF buffer (50 mmol/l Tris buffer pH 7.3, 250 mmol/l sucrose 1 mmol/l phenylmethylsulfonyl fluoride [PMSF]) and 1 mmol/l EGTA. This homogenate was centrifuged for 10 min at 10,000g; the cytosol was then centrifuged for 1 h at 100,000g, and the pellet was resuspended in 1 ml of Tris-sucrose-PMSF buffer. The resuspended pellet was incubated at 4°C for 30 min in the presence of triton X-100 at a final concentration of 0.1%. Protein content was measured by the Bio-Rad assay (Bio-Rad, Hercules, CA), using bovine serum albumin as the standard. Equal amounts of protein (20 µg) were subjected to a 10% SDS-PAGE and

TABLE 1

Steady-state plasma glucose, FFA, insulin and glucagon concentrations during the in vivo studies

| Group | Glucose (mmol/l) | FFA (mmol/l) | Insulin (µU/ml) | Glucagon (pg/ml) |
|---------|------------------|-------------------|-----------------|------------------|
| Control | 7.2 ± 0.2 | 0.56 ± 0.09 | 39 ± 5 | 148 ± 10 |
| FFA (+) | 7.0 ± 1.3 | $1.59 \pm 0.12^*$ | 41 ± 6 | 143 ± 15 |
| FFA (-) | 7.1 ± 0.2 | $0.26 \pm 0.07^*$ | 38 ± 5 | 156 ± 16 |

Data are means \pm SE. Plasma was sampled at 30-min intervals during the last 3 h of the in vivo studies for the determination of FFA, insulin, and glucagon concentrations. Three groups of conscious rats were studied: 1) control rats (control) receiving saline infusions during islet clamp studies, 2) rats receiving nicotinic acid and intralipid infusion for 3 h [FFA (+)], and 3) rats receiving nicotinic acid infusion alone for 3 h [FFA (-)]. Plasma was sampled at 10 min intervals for the determination of plasma glucose concentrations. * $P < 0.01$ vs. control.

electrophoretically transferred to nitrocellulose membranes. After blocking, the membranes were incubated with a 1:2,500 dilution of polyclonal anti-Glc-6-Pase antibody (provided by Dr. Rebecca Taub, University of Pennsylvania), followed by a 1:5,000 dilution of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody kit (Amersham, ECL, Arlington Heights, IL).

Northern blot analysis. Total RNA was isolated from freeze-clamped liver tissues according to the RNA-STAT kit (Tel-TEST "B", Friendswood, TX). The isolated RNA was assessed for purity by the 260:280 absorbency ratio. Total RNA (20 µg) was electrophoresed on a 1.2% formaldehyde-denatured agarose gel in $1 \times$ MOPS running buffer. The RNA was visualized with ethidium bromide and transferred to a hybond-N⁺ membrane (Amersham, Arlington Heights, IL). We used a 1.25-kb *Eco-HindIII* Glc-6-Pase cDNA (provided by Dr. Rebecca Taub), which was labeled with [α ^{32}P]dCTP, using the Megaprime labeling system kit (Amersham). Prehybridization was performed for 4 h at 42°C in $5 \times$ SSC, 50% formamide (vol/vol), $5 \times$ Denhardt's, 100 mg/ml salmon sperm DNA, and 0.1% SDS. Hybridization was carried out for 16 h in the same buffer with the ^{32}P -labeled probe. The filters were washed three times for 10 min in $2 \times$ sodium chloride-sodium citrate (SSC)/0.1% SDS at room temperature and twice in $0.1 \times$ SSC/0.1% SDS for 30 min at 55°C . They were then exposed to Fuji X-ray film for 12-48 h at -80°C with intensifying screens. Quantification was done by scanning densitometry.

Analytical procedures. Plasma glucose was measured by the glucose oxidase method (Glucose Analyzer II, Beckman Instruments, Palo Alto, CA), and plasma insulin and glucagon were measured by radioimmunoassay. Plasma FFA concentrations were determined by an enzymatic method with an automated kit according to the manufacturer's specifications (Waco, Osaka, Japan). Differences between groups were determined by analysis of variance.

RESULTS

Biochemical parameters during the pancreatic clamp studies. The infusion of somatostatin and insulin (control) raised the plasma insulin concentration by $\sim 50\%$ above fasting levels to an average of $\sim 40 \text{ µU/ml}$. The plasma insulin concentrations were similar among the three experimental groups (Fig. 1A). Similarly, the average plasma glucagon concentrations during the in vivo studies were not significantly different in the three experimental groups (Table 1). The plasma glucose levels (Fig. 1B) were maintained at an average of $7.1 \pm 0.7 \text{ mmol/l}$ during the clamp studies in all groups. To maintain the plasma glucose concentrations at their basal levels, it was necessary to infuse exogenous glucose at rates of 2.4 ± 0.7 , 9.4 ± 2.2 , and $0.7 \pm 0.3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ in the control, nicotinic acid, and FFA groups, respectively. The plasma FFA concentrations declined modestly in the presence of mild hyperinsulinemia in the control study (Fig. 1C). However, the nicotinic acid infusion decreased the plasma FFA levels markedly

Glc-6-Pase (mRNA)

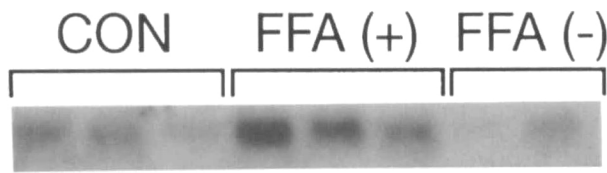


FIG. 2. Effect of hyperlipidemia on hepatic Glc-6-Pase mRNA. Northern analysis of Glc-6-Pase mRNA was performed in liver freeze-clamped *in situ* at the completion of the pancreatic clamp studies. Total RNA (20 μ g) of each liver RNA sample was loaded on each lane, and equal loading was confirmed by ethidium staining of the 18S and 28S ribosomal RNA bands. The blots were probed with [32 P]Glc-6-Pase cDNA. The figure depicts Glc-6-Pase mRNA in groups ($n = 3$) of samples obtained from control rats (CON), rats receiving nicotinic acid and intralipid infusion for 3 h [FFA (+)], and rats receiving nicotinic acid infusion alone for 3 h [FFA (-)]. Analysis was performed multiple times for each rat.

(by ~70%) from basal levels of 0.72 ± 0.05 . Finally, the combined infusion of intralipid/heparin and nicotinic acid raised the plasma FFA levels by approximately twofold from an average of 0.82 ± 0.08 during the basal period (Fig. 1C).

Effects of nicotinic acid and intralipid infusion on Glc-6-Pase mRNA abundance. As presented above, the experimental design allowed us to examine in conscious rats the impact of physiological changes in the plasma lipid concentrations on the expression of the Glc-6-Pase gene in the liver. The relative abundance of Glc-6-Pase mRNA was examined by Northern blot analysis using a 1.25-kb cDNA that recognizes the catalytic portion of this enzyme. Figure 2 shows that 3 h of hyperlipidemia markedly increased the Glc-6-Pase mRNA in the liver of nondiabetic rats. The regulatory role of plasma lipids on the hepatic abundance of Glc-6-Pase mRNA is also supported by the opposite effect of the inhibition of lipolysis by nicotinic acid. The comparison of the two groups receiving nicotinic acid with or without concomitant infusion of intralipid also allows one to discriminate the effects of the changes in the circulating concentrations of lipids from the potential effects of the lipid-lowering agent *per se*.

Effect of circulating lipids on hepatic Glc-6-Pase protein. To examine whether the lipid-induced increase in the mRNA coding for the catalytic portion of Glc-6-Pase was paralleled by a concomitant increase in the protein levels in liver microsomes, we performed Western blot analysis using a polyclonal antibody against the catalytic portion of Glc-6-Pase (23,32). As can be seen in Figure 3, in the presence of equal infusions of nicotinic acid, a marked (approximately threefold) increase in the catalytic portion of Glc-6-Pase protein was detected after 3 h of hyperlipidemia. Thus, the increase in mRNA was accompanied by a similar increase in the Glc-6-Pase protein levels, suggesting an increase in transcription of the Glc-6-Pase gene in response to increased lipid availability.

DISCUSSION

The purpose of this study was to examine whether changes in the hepatic availability of lipids participate in the regulation of the Glc-6-Pase gene in the liver. While the potential role

Glc-6-Pase (protein)

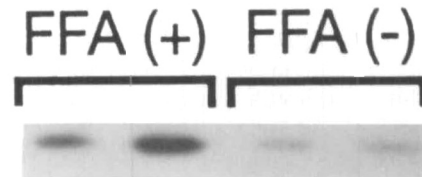


FIG. 3. Effect of hyperlipidemia on hepatic Glc-6-Pase protein. This figure shows an immunoblot of the liver microsomal fraction of rats receiving nicotinic acid and intralipid infusion for 3 h [FFA (+)] and rats receiving nicotinic acid infusion alone for 3 h [FFA (-)]. 20 μ g of microsomal protein was subjected to a 10% SDS-PAGE transfer to nitrocellulose membranes and blotted with antibodies against the catalytic unit of Glc-6-Pase. Samples are from rats that were killed and liver freeze-clamped *in situ* after 3 h at the desired plasma FFA levels (as in Fig. 1). Analysis was performed multiple times for each rat.

of circulating lipids in regulating the expression of hepatic enzymes has generally been examined *in vivo* using dietary manipulations of fat intake (16), we believe that such an experimental approach may fail to address the direct role of circulating lipids on Glc-6-Pase mRNA and protein. In fact, the complex regulation of the hepatic expression of this enzyme includes potent effects of insulin, cAMP, dexamethasone, and glucose (21–23,32,33), which all may be altered by dietary manipulations. Thus, we used nicotinic acid and lipid infusions to generate various concentrations of circulating lipids while controlling other hormonal and metabolic parameters. Our findings indicate that changes in the plasma lipid concentrations within the physiological range modulate the level of expression of the Glc-6-Pase gene in the liver of conscious rats.

In particular, the hepatic expression of Glc-6-Pase was decreased by the inhibition of lipolysis with nicotinic acid and induced by the infusion of a lipid emulsion. Because Glc-6-Pase is the final step of both the gluconeogenic and glycogenolytic pathways of glucose release in the liver, this observation may have implications for the pathophysiology of increased hepatic glucose output in the presence of sustained hyperlipidemia. It also suggests that the mechanism(s) by which a sustained increase in the availability of lipids might affect hepatic glucose output involves more than the short-term increase in the rate of gluconeogenesis (15). Indeed, increased hepatic glucose production is an early (~1 week) consequence of high-fat feeding in rodents (34), and the plasma FFA levels are closely related to the rate of endogenous glucose production in humans (8–10,12,13). Furthermore, most patients with NIDDM display increased concentrations of plasma FFA throughout the day (14).

While the pathophysiological relevance of our findings to metabolic conditions characterized by increased hepatic release of glucose and hyperlipidemia is intriguing, it may appear less obvious how this regulatory mechanism may participate in the normal homeostasis of postprandial carbohydrate metabolism. Indeed, the mRNA and protein levels of Glc-6-Pase are markedly suppressed by insulin (21–23,33),

while glucose (32) and lipids (present study) tend to induce the expression of this enzyme. Thus, the combined effect of dietary carbohydrates and lipids may buffer the effect of postprandial hyperinsulinemia on the hepatic expression of Glc-6-Pase. This may in turn prevent an excessive decline in the enzyme expression during the transition from the postabsorptive to the fasting condition, when increased Glc-6-Pase activity is necessary to prevent hypoglycemia. It may also be argued that the balance between hepatic insulin action and postprandial levels of lipids and glucose may have a major impact on the level of expression of Glc-6-Pase during the postabsorptive and fasting periods. Whether the induction of hepatic Glc-6-Pase expression by glucose (32) and lipids contributes to the increased rate of hepatic glucose production that follows a high-carbohydrate (35) and high-fat diet (34) remains to be delineated.

The molecular mechanism by which increased lipid availability increases the mRNA and protein levels of Glc-6-Pase in the liver cannot be determined by the present study. However, recent reports have demonstrated regulation of several genes by lipids through posttranscriptional (36) and transcriptional mechanisms (20,37). The latter have been suggested to be mediated via the peroxisome proliferator-activator receptors (20,37).

In conclusion, we demonstrate that plasma lipid concentrations modulate the level of expression of Glc-6-Pase in vivo. It is suggested that prolonged hyperlipidemia may contribute to the increased production of glucose via increased expression of this protein. Taken together with numerous other reports on the impact of lipids on carbohydrate metabolism in skeletal muscle (7-9), liver (8-10,12,13), and pancreatic β -cells (11,38,39), our finding provides experimental support for the role of hyperlipidemia in the pathogenesis of NIDDM (40,41).

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