Liver Glucose-6 Phosphatase Activity Is Inhibited by Refeeding in Rats 1,2

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ABSTRACT This study was conducted to determine whether inhibition of hepatic glucose-6 phosphatase is involved in the mechanism of suppression of hepatic glucose production during the postprandial period. We studied the time course of changes in the enzyme activity by refeeding food-deprived rats with a nonpurified diet. The Vmax of the enzyme, assayed in homogenates from livers of unfed rats (12.3 ± 0.15 U/g wet liver, mean ± SEM, n = 6) was progressively decreased upon refeeding: 11.1 ± 0.5, 8.5 ± 0.4 and 7.9 ± 0.5 U/g, in rats refeed for 90, 180 (P < 0.01) and 360 min (P < 0.01), respectively. The Km of the enzyme was not affected by refeeding. No inhibition of the enzyme was observed in microsomes purified from these homogenates, suggesting a metabolite-induced inhibition mechanism. To assess the role of insulin in the inhibition, we assayed the glucose-6 phosphatase activity in similarly processed liver homogenates from food-deprived rats perfused with insulin at physiological and supraphysiological concentrations, whereas plasma glucose was maintained at the basal level by adapted glucose perfusion (euglycemic clamps). No inhibition of glucose-6 phosphatase was found under these conditions, suggesting that insulin cannot by itself account for the inhibition observed in the refeeding experiments. These data constitute the first demonstration of the inhibition of glucose-6 phosphatase activity during the postprandial period. J. Nutr. 125: 2727-2732, 1995.

INDEXING KEY WORDS:
• glucose-6 phosphatase • liver • refeeding • insulin • rats

Catalyzing the last common step of both glycogenolysis and gluconeogenesis, glucose-6 phosphatase (G6Pase) [EC 3.1.3.9] hydrolyses glucose-6 phosphate (G6P) and allows the liver to release glucose. Because of its position in glucose metabolism, the G6Pase flux is a major determinant of the hepatic glucose output. Ten years ago, the inhibition of G6Pase activity was suggested to take place after glucose ingestion (Newgard et al. 1984), but biochemical evidence has been lacking. The knowledge of such mechanism could represent a major breakthrough in the understanding of the insulin resistance and elevated hepatic glucose production occurring in Type 2 diabetes. These phenomena could be accounted for by an increase of the G6Pase flux (Efendic et al. 1985, 1988). The loss or the major impairment of regulation mechanisms of G6Pase activity could explain at least in part such an increase in glucose production.

Since Newgard et al. (1984) have provided the first indirect evidence that G6Pase should be inhibited after intragastric administration of glucose in food-deprived rats, the idea that the inhibition of this enzyme could be involved in the inhibition of hepatic glucose output has received further support. Particularly, several studies (Barzilai and Rossetti 1993, Bode et al. 1992, Christ et al. 1986, Gardner et al. 1993, Minassian et al. 1994), have all shown the quantitative discrepancy between the measured global glucose output flux, which is strongly suppressed, and the calculated theoretical flux through G6Pase (estimated from the in vitro determination of the kinetics of the enzyme and the in vivo concentration of its substrate), which is not or is only slightly altered. These studies have led to the conclusion that a mechanism of inhibition of G6Pase, which could be dependent on a metabolite...
lost or diluted under in vitro conditions of assay, should take place in situ. However, because the inhibition is not observable during G6Pase assay, it has remained a matter of controversy.

The aim of the present work was to determine whether G6Pase activity is inhibited under conditions of inhibited glucose production. We studied two experimental models of inhibition of hepatic glucose output, i.e., food-deprived rats refed with nonpurified diet or perfused with insulin while glycemia was maintained at basal level by glucose perfusion (hyperinsulinic euglycemic clamp). In addition, we assayed the enzyme in homogenates after very rapid processing of portions of livers freeze-clamped in situ in anesthetized animals; this procedure allowed us to best retain the metabolic state of the liver.

MATERIALS AND METHODS

Refeeding experiments. Male Sprague Dawley rats (IFCA CREDO, L’arbrésle, France) weighing 180–200 g were housed for 3 d with free access to water and nonpurified diet [starch, 500 g/kg diet; proteins, 235 g/kg; lipids, 50 g/kg; cellulose, 40 g/kg; mineral salts, 55 g/kg; water, 120 g/kg (U.A.R. Epinay-sur-Orge, France)]. Rats were then deprived of food for 48 h with free access to water. Unfed rats were anesthetized by a single injection of pentobarbital (7 mg by 100 g of body weight). After 15 min, the abdomen was incised to expose the liver, which was then freeze-clamped in situ between steel blocks at liquid N2 temperature. The freeze-clamped liver was then removed and stored at −80°C. Blood was collected for the determination of plasma glucose (Bergmeyer et al. 1974) and insulin (Hales and Randle 1963). Refed rats were given free access to nonpurified diet for 90, 180 or 360 min. Food intake was not monitored. At the end of the appropriate feeding period, they were anesthetized for further processing of the liver as described above. The results were obtained from two sets of experiments (each set involving one separate group of three rats at each time point), both yielding comparable results.

Hyperinsulinic euglycemic clamp. Unfed rats were anesthetized using a single intraperitoneal injection of pentobarbital (70 mg/kg). Polyethylene catheters were placed in the left carotid artery and in the contralateral jugular vein for blood sampling and insulin and glucose [or saline] infusions, respectively. Blood glucose was determined every 5 min using a Glucometer II (Bayer diagnostics, Puteaux, France). In rats perfused with insulin (Lilly France S.A., Saint-Cloud), glucose [1.67 mol/L] was infused at variable rates to maintain glycemia at its initial value. After 50 min, a laparotomy was performed to allow free access to the liver. The liver was frozen in situ and blood sampled as described above.

All animals used in this study were handled according to the recommendations of a local ethics committee for animal experimentation. Anesthetized animals were rapidly killed by heart excision just after liver and blood sampling.

Enzyme assays. Pieces of freeze-clamped livers were taken randomly to a total weight of ~200 mg, reduced in powder at liquid N2 temperature and rapidly homogenized by sonication in 0.25 mol/L sucrose, 10 mmol/L Heps, pH 7.4. Glucose-6 phosphatase was immediately assayed (in 20 mmol/L Tris-HCl, pH 7.3 for 10 min at 37°C) by complex formation of inorganic phosphate (Pi) liberated from G6P (1–20 mmol/L) and subtracting the amount of Pi produced from paratrophophosphatase (1–20 mmol/L) under the same conditions of assay, as previously described (Minassian et al. 1994, Minassian and Mithieux 1994). The phosphatase activity toward paratrophophosphatase, which we call paratrophophosphatase (PNPase), allowed us to estimate the activity of non specific phosphatases. The G6Pase kinetic parameters (Km and Vmax) were determined from the reciprocal plots of the velocity vs. the G6P concentration. The results are expressed as units [U] of enzymatic activity per gram wet liver at the Vmax: 1 U converts 1 μmol of substrate per minute under the conditions used. Mannose-6-phosphatase (M6Pase) was determined in the same manner from the hydrolysis of mannose-6-phosphate. Microsomes were purified from the same homogenates by differential sedimentation (Minassian et al. 1994, Minassian and Mithieux 1994). G6Pase was assayed in microsomes as described above. Microsomal G6Pase activity is expressed as 1 μmol of substrate hydrolyzed per minute per milligram protein. The latency of M6Pase, i.e., the percentage of total M6Pase activity revealed by detergent treatment, was estimated from the hydrolysis of 1 mmol/L mannose-6-phosphate by microsomal preparations treated or not treated for 20 min at 4°C in the presence of 5 g/L cholate. Glycogen was assayed according to Keppler and Decker (1974). Protein was assayed by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Statistical analysis was performed by ANOVA. When significance was established, the differences between individual groups of data were tested for significance using Fisher’s test (Winer 1970). Values in the text are means ± SEM, n = 6.

RESULTS

Food-deprived rats had low plasma glucose and insulin. After 90 min refeeding, plasma glucose and insulin were higher by factors of 1 and 3, respectively [Table 1]. No further significant elevation occurred thereafter. G6Pase activity (~12–13 U/g wet liver in the unfed rats) was progressively decreased with re-
feeding [Fig. 1]. This represented a 35–40% inhibition in rats refeed for 360 min. This was specific for the phosphatase activity toward G6P because PNPass activity did not differ significantly among groups [Fig. 1].

The refeeding period was characterized by glycogen deposition: 16.8 ± 1.4, 32.2 ± 1.3 and 41 ± 3.7 mg/g wet liver in rats fed for 90, 180 and 360 min, respectively, versus 1.4 ± 0.5 mg/g wet liver in the unfed rats. This induced rehydration of the liver, resulting in a moderate progressive decrease of the liver protein concentration. The differences relative to the unfed rats were not statistically significant. However, we also expressed the G6Pase activity as units per milligram protein to obviate this small variation. The G6Pase activity, expressed as units per milligram protein, was significantly lower in rats refeed for 180 and 360 min compared with unfed rats, whereas no significant difference in PNPass activity was observed.

The G6Pase Km values were approximately 2 mmol/L in unfed and refeed rats. We determined M6Pase activity in the same manner as described for G6Pase [see Materials and Methods]. M6Pase represented ~50% of G6Pase in homogenates from unfed and refeed rats [data not shown]. G6Pase was further assayed in microsomal preparations obtained from liver homogenates of unfed and 360-min–refed rats. The protein recovery was the same in both unfed and refeed rats, ~10% of total homogenate protein. The latency of M6Pase in isolated microsomes was 45 ± 4 and 49 ± 5% in unfed and refeed rats, respectively [mean ± SEM, not significant]. Km values [close to 2 mmol/L] and Vmax [close to 0.2 mmol G6P hydrolyzed per minute per milligram protein] of G6Pase in isolated microsomes from unfed and refeed rats were not different.

In the same manner, we determined G6Pase activity in the homogenates from livers, freeze-clamped in situ, of unfed rats perfused with insulin during a hyperinsulinic euglycemic clamp. The range of insulinemia studied covered plasma insulin concentrations observed during the refeeding experiments and supra-physiological plasma insulin concentrations. No significant inhibition of G6Pase was observed in any case, irrespective of the amount of insulin perfused (Table 2).

**TABLE 1**

<table>
<thead>
<tr>
<th>Refeeding time</th>
<th>Plasma glucose mmol/L</th>
<th>Plasma insulin pmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.3 ± 0.6</td>
<td>83 ± 14</td>
</tr>
<tr>
<td>90</td>
<td>11.1 ± 0.7*</td>
<td>366 ± 54*</td>
</tr>
<tr>
<td>180</td>
<td>10.1 ± 1.1*</td>
<td>331 ± 56*</td>
</tr>
<tr>
<td>360</td>
<td>9.2 ± 0.8*</td>
<td>345 ± 41*</td>
</tr>
</tbody>
</table>

1 Values are the means ± SEM (n = 6 per group), * significantly different from unfed rats (Fisher's test, P < 0.05).

**DISCUSSION**

In the present work, involving a freeze-clamping approach, we have shown that the liver G6Pase activity is inhibited after refeeding unfed rats. It should be mentioned that in very similar preliminary experiments, involving very rapid processing of liver homogenates and/or purification of microsomes, but not prior freeze-clamping of the tissue, we could not demonstrate any inhibition of G6Pase within the same period of refeeding (Daniele, Minnassian and Mithieux, unpublished results).

Workers in the field of G6Pase are usually reluctant to use frozen tissues when studying this enzyme in isolated microsomes. It is indeed well known that one feature of the enzyme, i.e., M6Pase latency, is significantly different in microsomes isolated from frozen livers (~50% in the present study, see Results) and in

**FIGURE 1** Time course of hepatic glucose-6-phosphatase activity during refeeding previously unfed rats with nonpurified diet. G6Pase and PNPass were assayed, and G6Pase activity was calculated as described in Materials and Methods. The results are expressed as U/g wet liver. One unit of G6Pase converts 1 μmol of substrate per minute at 37°C under the conditions of assay. Values are expressed as means ± SEM [n = 6 per group]. * Significantly different from unfed value [P < 0.01, Fisher's test].
TABLE 2
Effect of insulin on glucose-6 phosphatase activity in unfed rats with euglycemic clamps1

<table>
<thead>
<tr>
<th>Insulin perfused (pmol/h)</th>
<th>Glucose perfused (μmol/min)</th>
<th>Basal glucose (mmol/L plasma)</th>
<th>Final glucose (mmol/L plasma)</th>
<th>Final insulin (pmol/L plasma)</th>
<th>Final G6Pase (U/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>11 ± 1</td>
<td>6.4 ± 0.2</td>
<td>6.8 ± 0.2</td>
<td>97 ± 7</td>
<td>12.0 ± 0.8</td>
</tr>
<tr>
<td>8</td>
<td>18 ± 2</td>
<td>6.4 ± 0.1</td>
<td>7.1 ± 0.2</td>
<td>221 ± 8</td>
<td>10.0 ± 0.4</td>
</tr>
<tr>
<td>4</td>
<td>35 ± 1</td>
<td>6.7 ± 0.5</td>
<td>6.8 ± 0.2</td>
<td>324 ± 29</td>
<td>11.3 ± 0.7</td>
</tr>
<tr>
<td>3</td>
<td>50 ± 2</td>
<td>6.0 ± 0.4</td>
<td>6.9 ± 0.5</td>
<td>704 ± 94</td>
<td>12.1 ± 1.2</td>
</tr>
<tr>
<td>3</td>
<td>49 ± 2</td>
<td>5.9 ± 0.2</td>
<td>6.0 ± 0.2</td>
<td>&gt;1400</td>
<td>14.6 ± 0.8</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM (n = the number of animals indicated).
2 Glucose perfused was the mean infusion rate at the end of the clamp [adjustments of this rate was never required during the last 20 min].
3 Basal plasma glucose was determined from a 100-μL blood sample taken just before perfusion.
4 Final plasma glucose and insulin concentrations were determined using the final blood sample.
5 One unit of G6Pase converts 1 μmol of substrate per minute at 37°C under the conditions of assay.
6 ANOVA did not reveal any significant difference among the different groups, P > 0.15.

those obtained from fresh livers (~90%, Minassian et al. 1994, Minassian and Mithieux 1994). The lower M6Pase latency in microsomes isolated from frozen livers, which means that substantial M6Pase activity is expressed in the absence of detergent treatment, can be differently interpreted according to the two main structural models for G6Pase organization within the membrane (Sukalski and Nordlie 1988). For the defenders of the substrate transport model, which postulates that G6Pase activity results from the combined action of a transmembranous G6P transporter and a nonspecific phosphohydrolase located inside the lumen of microsomes, a low M6Pase latency means that a substantial proportion of membranes has been ruptured by the freezing procedure. According to the supporters of the alternative conformational model, it reveals only a different conformation of the enzyme or different enzyme-membrane interactions, having repercussions on its enzymatic characteristics, among them a decreased specificity towards G6P. We would like to point out that the problem of the different M6Pase latencies in microsomes from frozen versus fresh livers does not arise when G6Pase is rapidly assayed in homogenates before the purification of microsomes. It has indeed been clearly shown that the latency of M6Pase in situ in isolated hepatocytes prepared from fresh livers is considerably lower (54%) than in purified microsomes isolated from fresh livers, in which M6Pase is ~90% latent [Jorgenson and Nordlie 1980, Nordlie and Jorgenson 1981]. The latency of M6Pase in situ in fresh livers is therefore comparable with the latency of M6Pase in microsomes isolated from frozen livers (~50%). This is in keeping with the observation that total M6Pase activity also represents ~60% of total G6Pase activity in such hepatocytes from fresh livers [Jorgenson and Nordlie 1980]. These data are in agreement with those observed with homogenates from fresh livers in this laboratory [Daniele, Minassian and Mithieux unpublished results, see above]. Therefore, regarding the G6Pase: M6Pase ratio, the data presented here using homogenates from frozen livers exhibiting a M6Pase activity equal to ~50% of G6Pase, should be more representative of G6Pase activity in situ than G6Pase activity assayed in intact microsomes isolated from fresh liver. Moreover, two recent reports involved G6Pase assay from frozen livers [Barzilai and Rossetti 1993, Burcelin et al. 1995].

We present biochemical evidence that G6Pase activity is inhibited after refeeding, confirming the previous indirect data of Newgard et al. [1984]. The role of insulin was not approached in this previous work. Because the rise of insulinemia follows nutrient availability during refeeding experiments, the actual role of insulin in G6Pase inhibition is therefore difficult to estimate. On the other hand, it can be estimated by insulin perfusion with euglycemic clamps [Terrettaz et al. 1986a]. G6Pase was not inhibited upon insulin perfusion in unfed rats. This was true even under conditions of maximal velocity of glucose utilization, which was clearly reached at the two highest doses of insulin perfused because a further increase of the amount of glucose perfused was not required to maintain glycemia [see Table 2]. However, the hepatic glucose output is totally suppressed within an insulinemia range much lower than that required to obtain maximal glucose utilization [Terrettaz et al. 1986b]. Taken together, the present data suggest that if insulin is involved in the inhibition of G6Pase after refeeding, it cannot account for the inhibition of G6Pase alone. Other factors must be required, either for the inhibition to be allowed to take place or to mediate the inhibition. The possible role of nutrients, which have also been shown to have an action in insulin secretion...
(Prentky and Matschinsky 1987), might be considered. Noteworthy, a mechanism of activation of glucokinase, the enzyme catalyzing the reverse reaction of G6Pase in the liver, has been recently demonstrated. It is dependent on a nutrient metabolite, fructose, and is independent of insulin (Davies et al. 1990, Van Schaftingen 1989, Van Schaftingen and Vanderkamp 1989).

The inhibition of G6Pase is characterized by a decrease of \( V_{\text{max}} \) and no alteration of \( K_m \), this suggests a noncompetitive inhibition mechanism. Two lines of evidence allow one to rule out the hypothesis that this decrease of the enzyme \( V_{\text{max}} \) could be due to a decrease in the amount of G6Pase protein: 1) the inhibition could not be demonstrated if processing of the liver does not involve freeze-clamping and 2) the inhibition does not persist in isolated microsomes. The latter seems to favor the hypothesis that an inhibitory metabolite is present in the homogenate and lost during the purification procedure. However, the fact that the inhibition does not persist in microsomes may also be in agreement with the hypothesis that there is a covalent (and labile) modification of the enzyme, or of a microsomal bound regulatory molecule, because isolation of microsomes takes ~3 h. Such covalent modification could involve phosphorylation-dephosphorylation mechanisms. The hypothesis that G6Pase activity could be regulated through phosphorylation mechanisms has already been supported by Begley and Craft (1981) but was contested later (Burchell and Burchell, 1982, Singh et al. 1983). In keeping with a hypothetical nutrient-dependent metabolite mechanism, we have previously shown that a glutamine metabolite, \( \alpha \)-ketoglutarate, in the presence of magnesium, is able to inhibit G6Pase in vitro at physiological concentrations (Minassian et al. 1994, Mithieux et al. 1990). We have also characterized the mechanism of inhibition of G6Pase by unsaturated fatty acids (Mithieux et al. 1993). More recently, we have shown that long-chain acyl-CoAs, which are involved as metabolic coupling factors in nutrient-induced insulin secretion (Prentky et al. 1992), inhibit G6Pase in vitro at physiological concentrations (Mithieux and Zitoun 1994). Interestingly, fructose-1 phosphate, the intracellular fructose metabolite responsible for the activation of glucokinase (Davies et al. 1990, Van Schaftingen 1989), has also been shown to inhibit G6Pase activity in vitro at high concentration (Robbins et al. 1991). Further work is currently in progress to determine precisely the nature of the inhibition and/or of the putative metabolite(s) involved in the inhibition evidenced here.

In conclusion, the data reported here constitute the first direct evidence for the inhibition of the activity of G6Pase during the postprandial period, a process that could participate in the inhibition of hepatic glucose production occurring in this situation. In addition, it is suggested that insulin cannot by itself account for the inhibition, which could be dependent on the presence of an inhibitory nutrient metabolite in the liver homogenate. These results establish the biochemical basis of the previous indirect data suggesting that G6Pase should be an important target of short-term control of hepatic glucose output in vivo.

**LITERATURE CITED**


