Communication

LIVER MICROSONAL GLUCOSE-6-PHOSPHATase IS COMPETITIVELY INHIBITED BY THE LIPID PRODUCTS OF PHOSPHATIDYLINOSITOL 3-KINASE*

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We have studied the effect of various phospholipids on the activity of glucose-6-phosphatase (Glc6Pase) in untreated and detergent-treated rat liver microsomes. Glc6Pase is inhibited in the presence of phosphoinositides in a dose-dependent manner within a range of concentration 0.5–10 μM. The order of efficiency in untreated microsomes is: phosphatidylinositol (PI) 3,4,5P3 > PI3,4P2 = PI4,5P2 > PI3P = PI4P > PI. In contrast, Glc6Pase is not inhibited in the presence of phosphatidylinositol 4-monophosphate; PI4,5P2, L-

Glucose-6-phosphatase (Glc6Pase) is a crucial enzyme of glucose homeostasis since it catalyzes the ultimate biochemical reaction of both glycolysis and gluconeogenesis, e.g. the hydrolysis of glucose-6-phosphate (Glc6P) in glucose and P1 (1). It allows the gluconeogenic tissues in which it is specifically expressed to release glucose in blood. Numerous data have been provided during the last years, strongly suggesting that Glc6Pase might play an important regulatory role in the glucose release by the liver and the kidney, through mechanisms involving either gene expression (2–4), and/or biochemical inhibition of its enzymatic activity (Refs. 5–7, recently reviewed in Ref. 8).

In contrast with most of the other regulatory enzymes of liver glucose metabolism, Glc6Pase is a very hydrophobic protein localized within the reticulum endoplasmic membrane (1, 8). This particular location within the cell is currently not explained. The observation that the kinetic properties of the enzyme are crucially dependent on the presence or the absence of the membrane has pointed out the importance of membrane lipids in the regulation of Glc6Pase activity in situ (8). In our previous works, we characterized the inhibition effect of unsaturated fatty acids (9) and of fatty acyl-CoA esters (10) on Glc6Pase activity. In the present work, we have addressed the question of the possible modulation of Glc6Pase activity by phospholipids themselves. Numerous previous studies have been devoted to the role of phospholipids in the stability and/or the activity of Glc6Pase by means of the effects induced by various phospholipases (11–15) or by phospholipid transfer proteins (15, 16). None has specifically addressed this question of the effect of the phosphorylation of the inositol ring of phosphatidylinositol. We have paid special attention to 3-phosphoinositides, the lipid products of phosphatidylinositol 3-kinase (PI3K) activity, since there is growing evidence to suggest that this enzyme is essential for insulin’s regulation of glucose metabolism (see Ref. 17 as a recent review).

EXPERIMENTAL PROCEDURES

Materials—L-α-Phosphatidylinositol (1-palmitoyl,2-linoleoyl) (PC), L-α-phosphatidylserine (PS), L-α-phosphatidylethanolamine (1,2-dioleoyl) (PE), L-α-phosphatidic acid (1,2-dioleoyl) (PA), diacylglycerol (1-stearoyl-2-arachidonoyl-sn-glycerol) (DAG), L-α-phosphatidylinositol 4-monophosphate (PI4P), L-α-phosphatidylinositol 4,5-diphosphate (PI4,5P2), L-α-phosphatidylinositol 3,4,5-trisphosphate (IP3), L-α-phosphatidylinositol 3-monophosphate (1,2-dipalmitoyl) (PI3,4P2), L-α-phosphatidylinositol 3,4-bisphosphate (1,2-dipalmitoyl) (PI3,4,5P3), L-α-phosphatidylinositol 3,4,5-trisphosphate (1,2-dipalmitoyl) (PI3,4,5P3) were obtained from Sigma (La Verpillière, France). L-α-Phosphatidylinositol 3-monophosphate (1,2-dipalmitoyl) (PI3,4P2), L-α-phosphatidylinositol 3,4-bisphosphate (1,2-dipalmitoyl) (PI3,4,5P3), and L-α-phosphatidylinositol 3,4,5-trisphosphate (1,2-dipalmitoyl) (PI3,4,5P3) were obtained from Matreya (Pleasant Gap, PA). PI3,4P2, and PI3,4,5P3 were also synthesized from PI, PI4P, and PI4,5P2 using PI3K immunopurified from human platelets and purified by thin layer chromatography as described previously (18). All lipids were stored in chloroform/methanol (1:1) under N2 at ~80 °C.

Preparation of Microsomes and Glc6Pase Assay—Microsomes were obtained from livers of 48-h fasted rats, as described previously (9). The term "untreated microsomes" refers to microsomes suspended in 10 mM Hepes, 0.25 mM sucrose, pH 7.4, without further treatment. "Detergent-treated" refers to microsomes which were treated in the presence of 0.5% (mass/volume) cholate for 20 min at 4 °C. Glc6Pase assay was performed in the presence of 20 mM Tris-HCl, pH 7.3 for 10 min at 30 °C (the Glc6Pase velocity was a linear function of time for 15 min under all conditions of substrate concentration). Lipids were dried under N2 and resuspended by sonication in 10 mM Tris-HCl, pH 7.3. They were added to microsomes previously diluted in the Tris buffer (see above). The reaction was immediately started by the addition of Glc6P (2.5–25 mM final concentration). P1 was determined by a complexometric method after the reaction had been stopped by addition of ascorbic acid/trichloroacetic acid (2%/10%, mass/volume) (19). To allow valuable comparisons, microsomes were present at a constant concentration (60 μM of...
**Competitive Inhibition of Glucose-6-phosphatase by 3-Phosphoinositides**

**RESULTS**

Glucose-6-phosphatase, assayed in untreated microsomes, was not affected in the presence of PE or PS within the range of concentration of 0.5 to 10 μM. In contrast, it was slightly inhibited in the presence of PI and P13P, and strongly inhibited in the presence of P13,4P2 and P13,4,5P3 within the same concentration range (Fig. 1). The inhibition was dependent on the phosphorylated state of the inositol ring: the higher the phosphate number, the more pronounced the inhibitory effect. In some instances, higher lipid concentrations (20 and 50 μM) were studied. The maximal inhibitory effect (75–80%) was reached from 5–10 μM for P13,4P2 (Fig. 1) and from 20 μM for P13,4,5P3 (not shown). The highest inhibitory effects were obtained at 50 μM for P13P and PI (about 60 and 40% inhibition, respectively). The relative efficiencies of phosphoinositides were compared at a concentration producing half-maximal inhibition of Glc6Pase activity (for P13,4,5P3), e.g. 2 μM. P13,4,5P3 was about 2.5 times more efficient than P13,4P2 and P13,4,5P3 were about 10–20 times more efficient than PI or P13P. PI4P and PI4,5P2 had similar inhibitory efficiencies as their respective isomers P13P and P13,4P2 (not shown). The inhibitory effect was the same whether microsomes had been preincubated in the presence of the inhibitors for 15 min at 25 °C or not. The dose-dependent inhibition effects were the same whether the inhibitors were added in the absence (Fig. 1) or in the presence of 20 μM PS (final concentration in the assay). Since the nature of the fatty acid esterified in positions 1 and 2 of glycerol might influence the inhibition effect (3-phosphoinositides (diplamitoyl) - from Matreya were synthetically obtained, D9-phosphoinositides were also synthesized in vitro from their natural precursors from Sigma (purified from bovine brain and likely enriched in arachidonic acid in position 2 of glycerol). However, both types of 3-phosphoinositides had very similar effects and the results presented in Fig. 1 constitute a mixing of both sets of results. Other compounds were tested up to concentrations of 100 μM: neither PE, PS, PC, nor DAG or IP3 (e.g. the products of the hydrolysis of P14,5P3 by phospholipase C) inhibited Glc6Pase (not shown). In contrast, PA slightly inhibited Glc6Pase to the same extent as P1 (not shown). After detergent treatment of microsomes, Glc6Pase was less sensitive to the inhibition by PI3,4,5P3. The enzyme was inhibited by 50% at 10 μM (not shown). In addition, there was no clear difference with the inhibition effect induced by PI3,4P2 and PI4,5P2 (see below).

**DISCUSSION**

In this paper, we report that Glc6Pase is specifically inhibited in vitro in the presence of minute amounts of a particular class of phospholipids, e.g. phosphoinositides. In contrast, it is unaffected upon addition of the other major phospholipids of the membrane, even at high concentrations. Noteworthy, the most potent effector is PI3,4,5P3, the major product of PI3K activity (20, 21), which is three times more efficient than its lipid precursor (PI4,5P2) to inhibit Glc6Pase in untreated microsomes. Although microsomes constitute artificially revesiculated membranes, in which some specific properties of Glc6Pase could have been lost, as compared with Glc6Pase assayed in homogenates from livers freeze-clamped in situ (22) or in filipin-permeabilized isolated hepatocytes (23, 24), one may suppose that they represent an acceptable environment to characterize in vitro the inhibition mechanisms of Glc6Pase under a conformational state close to that in vivo. That the enzyme is less efficiently inhibited by PI3,4,5P3 after detergent solubilization of the reticular membrane, and has lost was given by the abscissa of this point. The results were obtained from three different preparations of microsomes and are expressed as means ± S.D.

![Figure 1: Effect of phospholipids on Glc6Pase activity in untreated microsomes. Dose-response study in the range of concentration 0.5–10 μM. Untreated microsomes (60 μg of protein/ml) were incubated for 10 min at 30 °C in the presence of 1 mM Glc6P and of the indicated phospholipids. The results are the means ± S.D. of five (●), six (○), four (■), six (□), and three (● and ○) experiments. They are expressed as percent of control activity measured in the absence of added phospholipid (0.12 ± 0.01 μmol of Glc6P hydrolyzed per min/mg of protein). Protein assay with bovine serum albumin as a standard in all experiments.

| Phospholipid (μM) | Glc6Pase Activity (% of control) |
|-------------------|---------------------------------|
| 0                 | 100                             |
| 1                 | 80                              |
| 2                 | 60                              |
| 5                 | 40                              |
| 10                | 20                              |

**Table I: Inhibition parameters of Glc6Pase by phosphoinositides**

| Phospholipid | K<sub>i</sub> (μM) |
|--------------|-----------------|
| P14,5P<sub>3</sub> | 4.7 ± 0.8       |
| PI3,4P<sub>2</sub> | 5.0 ± 0.7       |
| PI3,4,5P<sub>3</sub> | 1.7 ± 0.3       |

<sup>a</sup> Significantly different from both P14,5P<sub>3</sub> and PI3,4P<sub>2</sub> values, p < 0.01 (Fisher’s test).

<sup>b</sup> Significantly different from corresponding value in untreated microsomes, p < 0.01.

The inhibition effects were the same whether the inhibitors were added in the presence of 1 mM Glc6P and of the indicated phospholipids. The results are the means ± S.D. of five (●), six (○), four (■), six (□), and three (● and ○) experiments. They are expressed as percent of control activity measured in the absence of added phospholipid (0.12 ± 0.01 μmol of Glc6P hydrolyzed per min/mg of protein).
different types of structural conformations, which could be related to the surrounding lipidic environment (8). Whether microsomes had been treated by detergent or not, the mechanism of inhibition of Glc6Pase by PI3,4,5P3, PI3,4P2, and PI4,5P2 is purely competitive. This strongly suggests that the substrate site is involved as a site of interaction of the inhibitors. This is not unexpected given the obvious structural analogy between the phosphorylated inositol ring and Glc6P. However, it is important to note that the lipidic part of the molecule is required for this interaction to take place, since IP3 has no inhibitory effect alone, even at concentrations by 10 times higher. This suggests that the association of the lipidic part of the inhibitors to some hydrophobic regions of the enzyme (either on the catalytic unit or on putative associated polypeptide(s) might stabilize the interaction between the phosphorylated ring and the catalytic site. This should strongly favor the formation of the enzyme-inhibitor complex ($K_I$ values are in the micromolar range) with regards to the enzyme-substrate complex ($K_M$ is in the millimolar range). That the $K_I$ values for the enzyme in detergent-treated microsomes are slightly higher than the corresponding ones in untreated microsomes suggests that the enzyme-inhibitor interaction is weakened upon the conformational change induced by the removal of surrounding lipids (8). Noteworthy, it has been reported that the treatment of rat liver microsomes by a phosphatidylinositol-specific phospholipase C induced a decrease of the Glc6Pase $K_M$ (14). This is in agreement with the elimination of a competitive inhibitor of the enzyme.

Numerous recent studies strongly suggest that PI3K is an essential enzyme in the signal transduction of insulin to glucose metabolism in various cell types (17). For example, there is a good correlation between the insulin's stimulation of PI3K activity and the glucose transport in isolated rat adipocytes (25) and between the inhibition of PI3K by specific inhibitors and the blockade of insulin stimulatory effects on glucose transporter translocation and glucose transport in 3T3-L1 (26) and isolated (27) adipocytes and in cultured myocytes and rat muscle (28). Also, insulin’s stimulation of PI3K activity is strongly reduced in the insulin resistant liver of the diabetic ob/ob mouse (29). The availability of synthetic analogues of 3-phosphoinositides has allowed to identify recently a variety of binding protein (see Ref. 30 as a review). Among them, different isoforms of protein kinase C have been shown to be activated in the presence of PI3,4,5P3 (31–33). However, the present work is the first report of the inhibition by PI3,4,5P3 of a key enzyme in glucose metabolism. There has been compelling indirect evidence provided that the activity of this enzyme is negatively regulated by insulin (6, 8, 34). With regards to the intracellular location of Glc6Pase (endoplasmic reticulum), it has been strongly suggested that the specificity of insulin to stimulate glucose transport via PI3K activation in adipocytes is dependent on the subcellular targeting of PI3K to low density microsomes, from which glucose transporters translocate (35–37). This might obviate the physiological concern that total PI3,4,5P3 is estimated to be by 10 times less abundant than its lipid precursor PI4,5P2 in most stimulated cells (38), while having only a three times higher potency to inhibit Glc6Pase in vitro. If such a microsome PI3K-targeting process takes place in the liver upon insulin stimulation, the ratio of PI3,4,5P3 to PI4,5P2 could be substantially higher in the close vicinity of Glc6Pase than elsewhere in the cell. It is also possible that the difference in sensitivity between the respective effects induced by the two compounds be higher in situ than in isolated microsomes (see above). Another important difference between the in vitro and in vivo situation is the way of access of the inhibitor to the enzyme: outside the membrane (in vitro) versus inside the membrane (in vivo). The competitive inhibition mechanism of microsomal Glc6Pase by the main lipid product of PI3K reported here might therefore be of special biological meaning.

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