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AN ENDOGENOUS GLYCOGEN-ASSOCIATED COMPOUND MODULATES GLUCOSE-6-PHOSPHATASE ACTIVITY IN RAT LIVER MICROSOMES

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SUMMARY: Liver glycogen is closely associated with the endoplasmic reticulum, which contains the glucose-6-phosphatase enzyme system that catalyses the final step of hepatic glucose production. To examine whether this structural association has functional consequences, microsomes were isolated from 48 h fasted (n=6) and ad lib fed rats (n=3). Microsomes from fed rats had a higher glycogen content and lower enzyme activity than fasted rats. Overall, glucose-6-phosphatase activity was inversely proportional to microsomal glycogen content.

Partially purified rabbit or rat liver glycogen, at physiological relevant concentrations (10-100 mM glucose equivalents), added directly to either intact or Triton-disrupted microsomes from fasted rats significantly decreased glucose-6-phosphatase activity. Inhibitory activity was present in native liver glycogen prepared by sedimentation and could be dissociated from glycogen by ion-exchange and ultrafiltration.

These findings suggest that a low molecular weight (< 5000 D) compound closely associated with glycogen can modulate glucose-6-phosphatase and may have a physiologic role in the regulation of hepatic glucose production. © 1993 Academic Press, Inc.

INTRODUCTION: The glucose-6-phosphatase (EC 3.1.3.9) complex catalyses the final step in hepatic glucose production from both the gluconeogenic and glycogenolytic pathways. This enzyme complex has several functional components including a phosphohydrolase and translocases for glucose-6-P (T1), phosphate (T2) and glucose (T3) (3,6). Its activity is augmented by fasting (4), diabetes (11) and glucocorticoids (12,13) and diminished by feeding (16). However, the cellular or metabolic basis for these changes is not known.

In a previous report, rabbit and human liver glycogen were observed to inhibit glucose-6-phosphatase. Neither the physiologic significance nor the mechanism of glycogen's inhibitory action was defined (8). In the current study, we observed that the reported inhibitory effect of glycogen is attributable to a low molecular weight (<5000 D) compound closely associated with glycogen, which comprises < 3% of both commercially available rabbit liver glycogen and rat

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liver glycogen prepared in our laboratory. The potential role for this glycogen associated inhibitor in the regulation of glucose production is discussed.

METHODS: Male, 250-300 g, Sprague-Dawley rats were used as a source for liver microsomes. Microsomes were isolated as described by Mithieux et al. (9) and stored at -70°C until use. Glucose-6-phosphatase activity is stable under these conditions for several months (15). Glucose-6-phosphatase activity was measured at 35°C, pH 7.0, with Tris-HCl buffer in both untreated intact microsomes and triton permeabilized microsomes. The reaction was terminated by the addition of 2.4 M perchloric acid and the amount of inorganic phosphate liberated was assayed using the method of Ames (1). The activity in intact microsomes reflects the overall rate of entry of glucose-6-P into the microsomes, phosphohydrolase activity and efflux of phosphate and glucose from within the microsomes. The activity in permeabilized microsomes reflects only the activity of the intraluminal phosphohydrolase activity. Intactness of microsomes (assayed using 1 mM mannose-6-P as substrate) exceeded 95% and the activity in microsomes not treated with detergent was corrected based on the intactness measured with mannose-6-P (2). Microsomal protein was measured using the Bradford method (5).

Rabbit liver glycogen, oyster glycogen, glucagon, Dowex (chloride form, mesh size 200-400) and buffer reagents were purchased from Sigma Chemical Co. Rat liver glycogen was prepared by alkaline solubilization of rat liver followed by repeated ethanol precipitation and by deproteinization with 10% trichloroacetic acid. Glycogen was then dialyzed overnight against 400 volumes of deionized water and reprecipitated with ethanol and dried. In some experiments (n=4) glycogen (with some contaminating microsomes) was isolated from livers of fed rats by density centrifugation using a 3 step sucrose gradients. Livers of fed rats were homogenized in microsome isolation buffer (0.25 M sucrose, 10 mM Hepes, pH 7.4). Microsomes were isolated and layered over a 3 step (20%, 40% and 55%) sucrose gradient (total volume 30 ml). Samples were spun 3.5 hr at 100,000 g using a SW28 rotor. Glycogen (with contaminant microsomes) in the 40% sucrose and 40% / 55% interface was collected and used to examine the effect of native glycogen on glucose-6-phosphatase. Results are presented as average $\pm$ SEM.

RESULTS: The microsomal glycogen content was much higher in fed than fasted rats (7.97 ± 2.0 vs 1.44 ± 0.22 μmol/mg protein, $p < 0.01$). When the Vmax of glucose-6-phosphatase from both fasted (n=6) and fed (n=3) rats was plotted as a function of the microsomal glycogen content, an inverse relationship was observed (Fig. 1).

Since microsomes from fasted rats had high glucose-6-phosphatase activity, they were used in studying the effect of adding glycogen to microsomes. Table 1 illustrates the effect of adding increasing concentrations of chemically-prepared rat or rabbit liver glycogen on the activity of glucose-6-phosphatase. As little as 10 mM rat liver glycogen significantly inhibited glucose-6-phosphatase activity. Addition of glycogen to membranes, then freezing the mixture overnight (-70°C) (this is done to mimic the storage condition of microsomes from fed rats), produced a marked (up to 40-70%) inhibition, even at very low glycogen concentration in the final assay mixture (Table 1). Glycogen was effective in inhibiting both glucose-6-phosphatase activity in intact microsomes and phosphohydrolase activity in Triton X-100 treated microsomes. In agreement with a previous report (8), the source of the added glycogen clearly influenced its

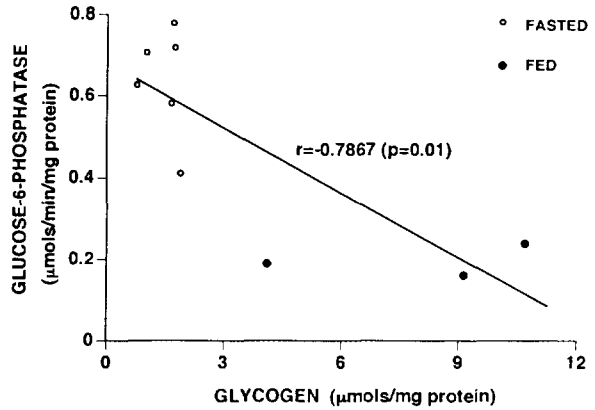

Fig. 1. Influence of microsome associated glycogen on glucose-6-phosphatase activity. Estimated value of glucose-6-phosphatase activity as a function of microsomal glycogen concentrations in 6 fasted and 3 fed rats.

effect on glucose-6-phosphatase activity. Rat liver glycogen was more potent than rabbit liver glycogen; oyster glycogen, at concentrations up to 100 mM, had no effect (data not shown).

To assess whether hexose chain length influenced this effect of glycogen, we treated rat liver glycogen with phosphorylase a (Sigma Chemical Co., nominally free of de-branching enzyme) for 60 min. This released approximately 40% of the glucose residues. Phosphorylase a treated glycogen has only short hexose chains beyond the α-1-6-branch points. This treated glycogen (100 mM), when mixed with microsomes and frozen overnight (-70°C), inhibited glucose-6-phosphatase by 75%, comparable to the effect of untreated rat glycogen. Likewise, treatment of glycogen with amyloglucosidase, which digested >80% of the glycogen, did not affect its inhibitory effect. These observations suggested the possibility that material which co-purified with glycogen, not glycogen per se, was responsible for the inhibition of glucose-6-

Table 1 Percent inhibition of microsomal glucose-6-phosphatase activity by added rabbit or rat liver glycogen

	20 min			Overnight	
	10 mM	50 mM	100 mM	2.5 mM	52.5 mM
Rabbit	11±6% (n=3)	25±3% (n=17)	51±9% (n=3)	24±2% (n=2)	25±8% (n=3)
Rat	23±7% (n=5)	30±7% (n=5)	48±7% (n=5)	43±2% (n=3)	63±4% (n=5)

Purified rabbit or rat glycogen was mixed with microsomes isolated from 48 h fasted rats. Microsomes were either: 1) incubated with 10, 50 or 100 mM glycogen for 20 min at 35°C prior to adding glucose-6-P to start the reaction, or 2) mixed with 100 mM glycogen and stored overnight at -70°C. In the microsomes frozen overnight with glycogen, microsomes were diluted into assay buffer containing either 0 mM or 50 mM glycogen, resulting in the final [glycogen] indicated in the table. The protein content in each assay mixture (total volume 1 ml) is ~100 μg.

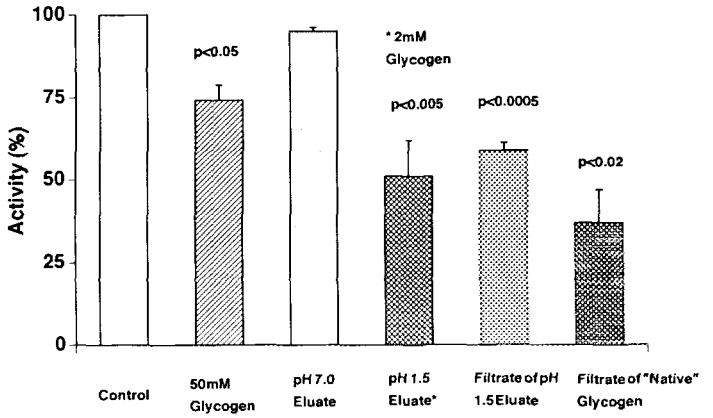

Fig. 2. Rabbit liver glycogen (pH 7.0) was passed through a Dowex anion exchange column. The glycogen portion which passed through the column (~95%) was referred to as pH 7.0 eluate. The portion eluted at pH 1.5 (contained ~5% glycogen) was referred to as pH 1.5 eluate. The final concentrations of glycogen in the glucose-6-phosphatase assay mixture are 50, 50, 2 and 0 mM respectively for 50 mM Glycogen, pH 7.0 Eluate, pH 1.5 Eluate and Filtrate of pH 1.5 Eluate groups. The filtrate of "native" glycogen is from 40 μmols of glycogen isolated by density centrifugation. Error bars indicate SEM of 3 experiments.

phosphatase. Purity of both rat and rabbit liver glycogen, assessed by recovery of glucose following amyloglucosidase digestion, was >97% on a weight basis. Inasmuch as dialysis, ethanol precipitation and trichloroacetic acid treatment did not remove the potential co-purifying inhibitor, we used a combination of anion-exchange chromatography and size exclusion by ultrafiltration to separate an inhibitory activity from glycogen. As shown in Fig. 2, 50 mM rabbit glycogen was passed through a 2.5x10 cm Dowex 1 (Sigma Chemicals) anion exchange column. That fraction (~ 95% of the added glycogen) not retained when the column is washed with water, did not inhibit glucose-6-phosphatase (Fig. 2). The residual glycogen bound to the column was eluted with 30 mM HCl and neutralized to pH 7.0. This material contained only ~5% of the glycogen but virtually all the inhibitory activity. This inhibitory activity was separated from residual glycogen by passage through an ultrafilter (nominal MW c.o. = 5000 D, Millipore Corp.) at pH 1.5. A similar inhibitory activity was found associated with rat liver, but not oyster glycogen. As with the addition of glycogen, addition of the partially purified inhibitor diminished glucose-6-phosphatase activity in both intact and disrupted microsomes to approximately the same extent (40-50%). This was seen in microsomes disrupted using either Triton X-100 (0.1%), deoxycholate (0.2%), or NH_4OH (0.1 M). Oyster glycogen had no inhibitory activity even when processed through the same treatment with KOH, ethanol, TCA, dialysis and ion-exchange procedures described. This suggested that the inhibitory activity arose from the tissue of origin of glycogen, and was not introduced during chemical isolation.

To further ascertain whether the inhibitory activity was present within the liver, glycogen was isolated from rat liver using a three step (20%, 40% and 55% sucrose) density gradient

centrifugation procedure. Glycogen was enriched in the 40% sucrose and 40% / 55% sucrose interface. This glycogen was pelleted and resuspended in microsome isolation buffer (0.25 M sucrose, 10 mM Hepes, pH 7.4). A very small amount of glucose-6-phosphatase activity from co-sedimented microsomes is present. When diluted (final glycogen concentration 10 mM) and added to microsomes from fasted rats, this glycogen (never treated with alkali, ethanol or TCA) inhibited glucose-6-phosphatase by 26±4 % (corrected for added glucose-6-phosphatase activity). In further experiments, glycogen prepared in this way was ultrafiltered (5000 MW cut-off) at pH 1.5 and the filtrate neutralized with NaOH. Compared with a buffer control that was acidified, filtered and neutralized in an identical manner, the filtrate from 40 μmols of glycogen inhibited (Fig. 2) glucose-6-phosphatase (0.07 mg microsomal protein) by 63±10 % (0.162±0.002 vs 0.062±0.02 μmol/min/mg protein at glucose-6-P concentration of 2 mM).

DISCUSSION: The inverse relationship between microsomal glycogen content and glucose-6-phosphatase might be a simple correlation without physiologic significance. At a minimum it indicates that when examining the regulation of glucose-6-phosphatase, investigators need to recognize that glycogen co-sediments with microsomes and the amount of glycogen present in the microsomes will, independent of other factors, affect glucose-6-phosphatase. The observation that addition of exogenous glycogen decreased glucose-6-phosphatase activity found in fasted rat liver microsomes down to values found in fed rats suggests a functional relationship. The demonstration that inhibitory activity was preserved when greater than 80% of the glycogen was digested by amyloglucosidase suggested that material other than glycogen was responsible for the inhibition observed. Separation of the inhibitor by a combination of ion-exchange chromatography and ultrafiltration provided further support for the presence of a compound which is closely associated with, but was distinct from glycogen. The observation that oyster glycogen prepared in an identical manner to rat and rabbit liver glycogen did not inhibit glucose-6-phosphatase suggests that the inhibitor is not introduced by the isolation process. This is further supported by the observation that glycogen separated from liver by density-gradient centrifugation, like that prepared using the chemical procedures, inhibits glucose-6-phosphatase.

The fact that both glycogen and the partially purified inhibitor affect glucose-6-phosphatase activity in both intact and permeabilized microsomes is intriguing. Other than the suggestion from the ultrafiltration studies that the inhibitor has a molecular weight <5000 D, neither its size nor chemical composition is known. Much evidence suggests that the active site of the phosphohydrolase component is accessed exclusively from the intravesicular side of the microsome and molecules as small as mannose-6-P are excluded (3,6). This suggests that either microsomes are selectively permeable to the inhibitor, or the inhibitor is acting on the cytosolic face of the microsome, indirectly affecting the phosphohydrolase. There is evidence that the

phosphohydrolase protein may span the membrane and be exposed to the cytosol (14). Alternatively, the inhibitor could associate with other elements of the glucose-6-phosphatase complex which are accessible to the cytosol (6) and affect the phosphohydrolase. The concentrations of glycogen (with associated inhibitor) that we and others (8) have found to inhibit glucose-6-phosphatase (10-100 mM) are well within the range found in liver cytosol from fed animals. In ad lib fed rats, liver glycogen concentration ranges roughly from 3-7 g/100 g liver (7). Estimating that the cytosolic compartment of liver parenchymal cells is less than 50 ml/100 g liver (10), this would correspond roughly to a minimum of 300 mM glycogen. Liver glycogen concentration in fasted rats is generally < 10 mM. Therefore, if a glycogen-associated inhibitor in the intact liver affects glucose-6-phosphatase in a manner similar to that observed in vitro, it may play a significant role in regulating flux through glucose-6-phosphatase.

In conclusion, the current studies demonstrate that glycogen, at physiological concentrations, inhibits glucose-6-phosphatase activity. This inhibition appears secondary to a low-molecular weight material which closely associates with glycogen both in the liver and throughout routine glycogen purification. Since glycogen co-sediments with glucose-6-phosphatase during routine microsome isolation, this glycogen-associated material may account for some of the difference in glucose-6-phosphatase activity previously reported among fed, fasted and diabetic rats.

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