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EFFECT OF ACUTE DIABETES ON RAT HEPATIC GLUCOSE-6-PHOSPHATASE ACTIVITY AND ITS MESSENGER RNA LEVEL

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SUMMARY: Glucose-6-phosphatase catalyzes the final step of glucose production by liver and kidney. Though its strategic position has sparked interest in its regulation, difficulty with isolating a pure, stable enzyme has slowed progress. Virtually all previous work examining the physiologic regulation of this enzyme has relied on estimates of glucose-6-phosphatase activity in crude microsome preparations. The recent cloning of human and murine glucose-6-phosphatase cDNAs has now allowed study of its mRNA expression. We studied the effect of acute, streptozotocin-induced diabetes on hepatic microsomal glucose-6-phosphatase activity and mRNA expression in young (89 ± 3 g), juvenile (304 ± 4 g) and adult (512 ± 10 g) rats. In control rats, mRNA expression and enzyme activity was similar among the three age groups. Streptozotocin-induced diabetes significantly increased the enzyme activities in both intact and triton-treated microsomes in all groups of rats ($p<0.001$). Glucose-6-phosphatase mRNA expression was increased in the diabetic rats as well ($p<0.0001$). Blood glucose concentrations correlated significantly with glucose-6-phosphatase mRNA level ($p<0.005$) and both intact ($p<0.002$) and triton-treated ($p<0.001$) microsomal glucose-6-phosphatase activity. Both intact and triton-treated microsomal glucose-6-phosphatase activity correlated with mRNA level ($p<0.001$, for each). We conclude that acute streptozotocin-diabetes increases expression of glucose-6-phosphatase mRNA and this contributes to the increased glucose-6-phosphatase activity seen with diabetes mellitus.

INTRODUCTION: A number of investigators have demonstrated that hepatic glucose-6-phosphatase (EC 3.1.3.9) activity increases in diabetic animals (1, 2). This increase in activity may, together with the increased activity of other hepatic enzymes involved in glucose production, contribute to the increased hepatic glucose output and hyperglycemia seen in diabetes (3, 4). The relative magnitude of the increase in glucose-6-phosphatase (fold-increase) is reportedly greater in adult compared to younger animals, in part due to a decline in basal activity with aging (5). Several recent studies in which glucose production was measured in vivo using tracer methods have provided further evidence that the increased glucose-6-phosphatase

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activity supports hepatic glucose production in fasted (6) and diabetic (7) rats despite lower concentrations of glucose-6-phosphate. In these and in the earlier studies cited, glucose-6-phosphatase activity (Vmax) was found to increase with no significant change in Km. This change was consistent with an increase in enzyme mass (2). However, the complexity of glucose-6-phosphatase (8) has made its isolation in a pure form difficult and no antibody or other method has been available for measurement of protein mass in liver (9).

In 1993 cDNAs and genes for murine and human glucose-6-phosphatase were cloned (10, 11). Sequence analysis of the deduced glucose-6-phosphatase indicates that they are hydrophobic proteins with an apparent molecular weight of 37 kD, a size comparable to that reported for the isolated phosphohydrolase (9, 12). There is ~89% amino acid homology between these species. To determine whether the changes in enzyme activity seen with diabetes reflect changes in mRNA expression and potentially protein synthesis, we measured the amount of glucose-6-phosphatase mRNA present in livers from young, juvenile and adult male control and diabetic rats using a murine glucose-6-phosphatase cDNA probe as previously described (11). The effects of age and diabetes on the mRNA levels were compared with the activity of the enzyme (both basal and detergent solubilized) from the same livers. The results suggest that increased expression or stability of glucose-6-phosphatase mRNA contributes to the increased glucose-6-phosphatase activity seen in liver of diabetic animals.

METHODS: Three groups of male Sprague-Dawley rats were studied. Young rats weighed 63-106 g, juvenile rats weighed 278-323 g and adult rats weighed 472-543 g. Rats were fed ad lib and maintained on a 12 hour dark/light cycle. Rats from each age group were rendered diabetic by intravenously injecting 100 mg/kg body weight of streptozotocin. Diabetes was confirmed by measuring blood glucose from the tail vein. Diabetic rats typically displayed polyuria, weight loss and increased blood glucose within 24 hours after streptozotocin injection. Control rats were given vehicle intravenously and sacrificed 3 days later. The diabetic rats were similarly sacrificed at the end of day 3. In the young and juvenile animals, additional rats were studied after the duration of diabetes was extended to 5 days by giving sufficient ultralente insulin (1-2 U/day) to slow or reverse wasting but not to correct glycemia. At the time of sacrifice all rats were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) and livers were excised. Part of the liver was quickly freeze-clamped in liquid nitrogen and used for RNA analysis, the remaining liver was used for microsome isolation and measurement of glucose-6-phosphatase activity.

Microsomes were isolated as described by Mithieux et al (13) and stored at -70°C until use. Glucose-6-phosphatase activity was measured in both untreated and Triton X-100 solubilized microsomes at 35°C, pH 7.0, in 50 mM Tris-HCl buffer with 10 mM glucose-6-phosphate. The reaction was terminated by the addition of 2.4M perchloric acid and the amount of inorganic phosphate liberated was assayed using the method of Ames (14). Microsomal protein concentrations were measured using the Bradford method (15).

Liver total RNA was extracted using a modified guanidine thiocyanate and CsCl centrifugation method (16) and stored at -70°C until use. To assess the purity and integrity of

the RNA isolated, total RNA was separated by electrophoresis in an agarose gel containing 2.2 M formaldehyde and transferred to a nylon membrane. The filter was hybridized with a 1091 bp cDNA probe containing nucleotides 730-1820 of the murine glucose-6-phosphatase, radiolabeled with ^{32}P-CTP by random priming (11). A single band was visualized (Fig. 1) as previously reported for mouse and human glucose-6-phosphatase (10, 11). To quantify glucose-6-phosphatase mRNA, a dot-blot assay was used. Initially, saturating amounts of cDNA were determined and thereafter, linearity of binding was verified by hybridizing hepatic RNA (0-40 μg) with an excess of cDNA. For this assay, 20 μg RNA is sufficient to provide a signal-to-noise of 5:1 in normal rats. All samples were counted simultaneously on a direct beta counter (Packard Instruments, Downers Grove, IL).

Inasmuch as severe diabetes changes the expression of multiple proteins in liver and total liver protein mass, the measured quantities of glucose-6-phosphatase mRNA were normalized to liver DNA content rather than to the expression of a housekeeping gene. For DNA analysis, liver samples were homogenized in Tris buffer (pH 7.5) and centrifuged at 12,000 g for 15 minutes. The pellets were resuspended in phosphate buffer and assayed for DNA content measured fluorometrically using bis benzimide. The RNA content of the same liver sample was measured and the RNA/DNA ratio for liver determined. This value was used to normalize the glucose-6-phosphatase signal to CPM/μg DNA. All data were expressed by ways of mean $\pm$ SE. Statistical comparisons among different treatment subgroups were performed using the ANOVA technique.

RESULTS: The mean blood glucose concentrations were not different among healthy rats of different ages (Table 1). Although both the intact and triton-treated microsomal glucose-6-phosphatase activity and glucose-6-phosphatase mRNA levels tended to be lower in adult compared to young and juvenile rats, the differences are not significant (Table 1 and Fig. 2).

Streptozotocin induced a significant increase of blood glucose concentrations at both day 3 and day 5 (Table 1). Streptozotocin-diabetes significantly increased glucose-6-phosphatase activity in both intact and Triton X-100 treated microsomes in all three age groups. Diabetes also significantly increased glucose-6-phosphatase mRNA levels in all three groups of rats. The changes in both glucose-6-phosphatase activity and its mRNA level occurred more abruptly in young and adult than in juvenile diabetic rats. In both Triton X-100 treated and intact microsomes, glucose-6-phosphatase activity correlated significantly with blood glucose concentration (Fig. 3). Likewise, there was a significant correlation between blood glucose and glucose-6-phosphatase mRNA content of the liver (Fig. 3). The glucose-6-phosphatase activity

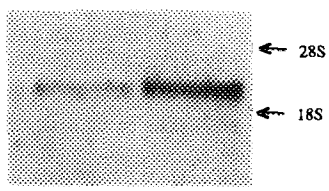

Fig. 1. Northern blot. First lane contains 10 μg and second lane contains 20 μg RNA.

Table 1. Effect of acute diabetes on body weight, blood glucose, and glucose-6-phosphatase activity in rats of different ages

	Body Weight		Blood Glucose	Glucose-6-Phosphatase (μmol/min/mg)	
	begin(g)	end (g)	(mmol/L)	Intact	Triton-treated
Young					
Control (n=4)	71±3		6.77±0.65	0.43±0.1	0.47±0.11
3 Day (n=9)	95±2	95±4	13.59±1.1	0.85±0.08	1.53±0.21
5 Day (n=4)	93±2	93±3	22.79±0.78	0.55±0.05	1.13±0.09
		ANOVA	P<0.0001	P=0.025	P=0.0016
Juvenile					
Control (n=4)	293±4		6.25±0.29	0.26±0.03	0.51±0.09
3 Day (n=4)	313±4	268±4	15.75±1.81	0.45±0.05	0.69±0.10
5 Day (n=3)	306±11	259±13	20.32±1.26	0.69±0.03	1.13±0.10
		ANOVA	P=0.0001	P=0.0002	P=0.0063
Adult					
Control (n=4)	495±11		6.46±0.14	0.25±0.03	0.30±0.04
3 Day (n=3)	536±4	468±7	15.4±1.15	0.54±0.04	0.91±0.04
		ANOVA	P=0.0003	P=0.0012	P=0.0002

of Triton X-100 treated microsomes, which is an index of the total enzyme present, was most highly correlated with glucose-6-phosphatase mRNA (Fig. 3).

Age had a significant effect on the expression of glucose-6-phosphatase activity in Triton X-100 treated microsomes ($P < 0.01$, ANOVA) in diabetic rats (Table 1). Glucose-6-phosphatase mRNA level in the diabetic rats, was similarly though less strongly affected by age ($P=0.067$, ANOVA).

DISCUSSION: These results provide the first clear evidence that streptozotocin-diabetes increases hepatic mRNA coding for glucose-6-phosphatase. They further confirm that diabetes increases the activity of glucose-6-phosphatase. The current results also indicate that there is a highly significant correlation between the increased activity and the increased mRNA content, particularly with the Triton X-100 treated microsomes which most closely report the phosphohydrolase activity. Taken together, these results indicate that increased expression of glucose-6-phosphatase is an important determinant of the augmented glucose-6-phosphatase activity seen in diabetes. The increased glucose-6-phosphatase activity was seen in all age groups, although both the activity and mRNA increases were most marked in the young and adult animals (Table 1 and Fig. 2). In the current study diabetes markedly increased the latency of the enzyme in the young (37% change, $p<0.0001$) and the adult (23% change, $p<0.001$). This has likewise been reported in previous studies of glucose-6-phosphatase kinetics in diabetes

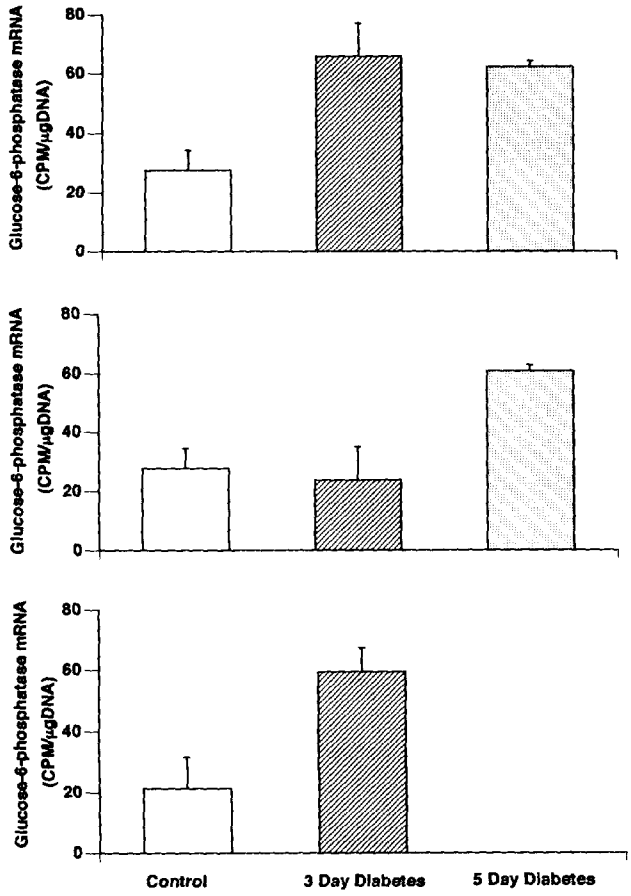

Fig. 2. Effect of acute streptozotocin-induced diabetes on glucose-6-phosphatase mRNA levels in young (upper panel), juvenile (middle panel) and adult (lower panel) rats. Statistical analysis was done using ANOVA. In each of the three age groups, the diabetes significantly increased glucose-6-phosphatase mRNA expression ($p < 0.0001$, ANOVA).

(2). Inasmuch as the cloned glucose-6-phosphatase appears to possess both glucose-6-phosphate phosphotransferase and phosphohydrolase activity (11), the increased latency seen with diabetes implies some differential regulation of these activities beyond the effect on mRNA expression described here.

In 2S FAZA hepatoma cells glucocorticoids can induce as much as a 10-fold increase in glucose-6-phosphatase activity and this is blocked by physiologic concentrations (10^{-10}M) of insulin (17). Interestingly, the latency of glucose-6-phosphatase is increased by glucocorticoid in these cells, again suggesting a greater effect on phosphohydrolase than on phosphotransferase activity. In FAO hepatoma cells, glucocorticoids were observed to increase mRNA expression coding for the phosphohydrolase (18) and this effect was blocked by high doses of insulin (1 μM). Whether glucocorticoids play a role in the induction of glucose-6-phosphatase mRNA in the insulin-deficient rats studied here will need to be addressed by further studies. At this time

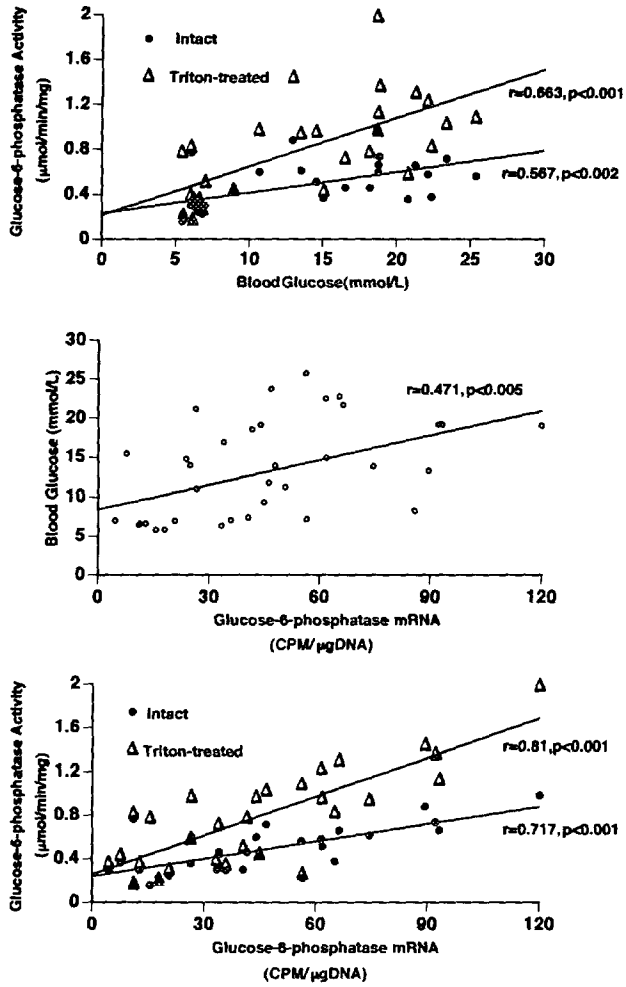

Fig. 3. Linear correlation analysis between blood glucose concentrations and glucose-6-phosphatase activity in both intact and Triton-treated microsomes (upper panel); between blood glucose and glucose-6-phosphatase mRNA levels (middle panel); and between glucose-6-phosphatase mRNA levels and glucose-6-phosphatase activity (lower panel).

it is not known, even in the cultured-cell system whether glucose-6-phosphatase transcription is being directly regulated by a glucocorticoid response element. In primary hepatocyte cultures hormones other than the glucocorticoids (thyroxine, epinephrine, glucagon) appear able to increase glucose-6-phosphatase activity (19). The transcriptional regulation of this gene may be rather complex, as has proved to be the case for other gluconeogenic regulatory enzymes (20). In this regard the effect of insulin to acutely inhibit glucose-6-phosphatase (6, 21) activity when added to rat liver microsomes likely occurs via a mechanism other than transcriptional regulation.

Although Triton X-100 stimulated glucose-6-phosphatase activity was observed to decline with age in these rats the effect was not nearly so dramatic as previously reported by Sen et al

(5). This was at least in part due to lower glucose-6-phosphatase activity in the healthy young rats studied here compared to those previously reported. Thus, we observe only a modest (< 2-fold) decline in phosphohydrolase activity and no significant change in mRNA between young and adult rats.

In conclusion, the current study implicates regulation of the expression of mRNA for the 37 kD glucose-6-phosphate phosphohydrolase as a major determinant of the increased glucose-6-phosphatase activity seen in experimental insulin-deficient diabetes. The increased glucose-6-phosphatase mRNA expression may contribute to the increased glucose production seen in uncontrolled diabetes. Further study is needed to define more fully the transcriptional regulation of glucose-6-phosphatase and the relationship of this to the acute regulation of glucose-6-phosphatase activity by insulin (6, 7).

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