ELSEVIER

Journal of Nutritional Biochemistry 12 (2001) 458–464

THE JOURNAL OF Nutritional Biochemistry

Diet and diabetic state modify glycogen synthase activity and expression in rat hepatocytes

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Received 6 July 2000; received in revised form 11 March 2001; accepted 21 March 2001

Abstract

Glycogen synthase (GS), a key regulatory enzyme in glycogen synthesis, is controlled by multisite phosphorylation and allosteric regulation and is activated by insulin. This study investigated changes in GS activity and expression in hepatocytes isolated from rats under altered nutritional and diabetic conditions. Experiments were carried out in healthy rats fed a chow diet, rats on high simple sugar (60% of energy from fructose and sucrose) or high fat (46% of energy from fat) diet, and in rats with streptozotocin induced diabetes. In the presence of insulin, activated GS activity (GS_I form) was increased by 89% in hepatocytes isolated from healthy rats. The stimulatory effect of insulin on GS activity and expression was blunted by cycloheximide and actinomycin treatment. In rats fed a high simple sugar or high fat diet, insulin stimulation of GS_I in isolated hepatocytes was impaired and GS expression was significantly lower in rats fed the high fat diet in comparison to controls. GLUT-2 protein expression was significantly lowered by both the high fat and high simple sugar diets. In hepatocytes isolated from diabetic rats, total GS activity (GS_T) was lower than in hepatocytes from healthy animals. Insulin added to the incubation medium did not stimulate GS activity, demonstrating impaired sensitivity to insulin in diabetic rats. However, insulin administration significantly increased GS expression indicating that a defect in synthase phosphorylation may be responsible for impaired GS activity in the diabetic state. The results presented in this study further confirm that GS activity is affected by both dietary and hormonal factors which can be measured in a rat hepatocyte model.

Keywords: Glycogen synthase; Rats; Hepatocytes; GLUT 2

1. Introduction

The biochemical pathways of liver glycogen metabolism are well-known and have been recently reviewed [1]. Hepatic glycogen synthase, the rate-limiting enzyme of the glycogen synthetic pathway, is controlled by intricate mechanisms involving multisite phosphorylation as well as allosteric regulation [2,3]. Insulin regulates blood glucose homeostasis by stimulating the utilization of glucose by liver, muscle and adipose tissue. In the liver, insulin stimulates glycogen synthesis, glycolysis and fatty acid synthesis, but unlike muscle and adipose tissue, it does not stimulate glucose transport. The conversion of glucose to glycogen in liver cells is dependent on the extracellular glucose concentration and on the presence of insulin, which stimulates glycogen synthesis over a wide range of glucose concentrations [4].

Hepatic glycogen synthase activity is thought to be increased in diabetic animals. However, the relationship between enzymatic activity, enzyme protein mass and mRNA content has not been well characterized. Gannon and Nuttall [5], as well as others have reported an increase in total synthase activity in liver from diabetic rats [6–9]. The increase in hepatic synthase activity could be due to an increased mass of the enzyme or to the presence of a more catalytically efficient form(s) of the enzyme. Although total activity may be enhanced in diabetic animals, the percent of activated glycogen synthase (GS_I), was significantly lower compared to hepatocytes from healthy control animals [5]. Conflicting data has been reported by other investigators. Rao et al. [10] reported no change in total glycogen synthase activity in livers of diabetic rats, but a significantly lower GS_I activity. Recently, Wang et al. [11] reported that both total GS and GS_I activity were significantly lower in livers of streptozotocin diabetic rats. In primary hepatocyte cul-

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0955-2863/01/$ – see front matter
PII: S0955-2863(01)00161-9

tures isolated from normal and diabetic adult rats it was shown that both glucose and insulin activate glycogen synthesis in primary culture hepatocytes from normal but not diabetic rats [12,13]. Van Auken et al. [14] reported that total GS activity in the diabetic cells at physiologic glucose concentrations was significantly higher than that for normal cells, however, the amount of active synthase was twofold lower than that of normal cells. Additionally, in normal hepatocytes, chronic (48 h) exposure to increasing concentrations of glucose was found to upregulate total synthase activity, synthase protein and synthase messenger RNA levels. All three of these responses were lost in hepatocytes from diabetic animals [14].

Data on the effect of dietary manipulation on glycogen synthase activity and expression in hepatocytes is limited. Kraegen et al. [15] have shown that rats fed with high fat diets developed peripheral and hepatic insulin resistance expressed by a decrease in peripheral glucose disposal and an increase in hepatic glucose uptake. Oakes et al. [16] demonstrated that compared with chow diet feeding, fat feeding significantly impaired insulin action in rats during euglycemic-hyperinsulinemic clamp. The insulin suppressibility of hepatic glucose production was decreased along with impaired insulin-stimulated glucose storage.

In the present investigation we have focused on possible changes in activity and expression of glycogen synthase in isolated hypatocytes under altered nutritional and diabetic conditions, based on previous experiments in adipose and muscle tissues indicating that dietary manipulation and diabetes may have a major impact on glycogen synthase activity.

2. Materials and methods

2.1. Experimental animals

Male rats (Sprague-Dawley, Anilab, Israel) were housed in light- and dark-cycled rooms (12 h light and 12 h dark) at a controlled temperature (24 ± 1°C) and fed a standard laboratory diet or designated diet with free access to food and water. In all experiments, animals were cared for under the guidelines set forth by the Animal Care Committee of the Hebrew University of Jerusalem, Israel.

2.2. Experimental procedures

Experiment 1: Healthy rats weighing 160–170 g, were anesthetized with Nemubutal (8 mg/100 g body weight) and hepatocytes isolated as described below. Liver cells were then incubated for 45 min with insulin (100 nmol/L), insulin + cycloheximide (50 ug/ml) or insulin + actinomycin (25ug/ml).

Experiment 2: Twenty one rats were divided into four groups such that average weight was similar (100–105 g) for each group. Rats were fed either a control diet, a high fat diet or a diet rich in simple sugars, for a 30 day period (Table 1). Following an overnight fast, blood samples were collected from tail tips for glucose and insulin measurements, animals anesthetized and hepatocytes harvested. Fresh cell suspensions were incubated with or without insulin for 45 min followed by determination of GS activity and expression and of GLUT-2 expression.

Table 1
Composition of experimental diets (g/100 g)

Component	Control	High simple sugar	High fat
Corn starch	60	—	7
Sucrose	—	30	—
Fructose	—	30	35
Oil	7	7	25
Casein	23	23	23
L-Methionine	0.3	0.3	0.3
Cellulose	5	5	5
Minerals[1]	3.5	3.5	3.5
Vitamins[2]	1	1	1
Kcal/100 g	395	395	485

[1] Mineral mix AIN 76A (ICN Biochemical, Cleveland, Ohio).
[2] Vitamin mix AIN 76A.

Experiment 3: Chemical diabetes was induced by a single intramuscular injection of streptozotocin (65 mg/kg bw; Sigma, Israel). Five days following the injection, plasma glucose levels were 12–15 mmol/L. Hepatocytes were isolated from diabetic rats and incubated with or without insulin **(**100 nmol/L**)** and with insulin and cycloheximide or actinomycin.

2.3. Preparation of hepatocyte homogenates

Hepatocytes were isolated based on the method of Seglen [17]. Rat livers were perfused with recirculating calcium-free HANKS buffer for 10 min (30 ml/min). Collagenase was then added to the perfusion medium (60 mg in 100 ml) and, after 10 min the cells were harvested and washed three times by centrifugation (500 × g) and resuspended in calcium-free buffer. Viability was 80–95% as determined by Trypan Blue staining. Cells were then incubated for 45 min and then homogenized for measurement of enzyme activity and protein expression.

2.4. Glycogen synthase assay

GS was assayed in hepatocytes using a modification of the methods of Thomas et al. [18] and Madar [19]. Fresh hepatocytes were homogenized in ice-cold buffer containing 10 mmol/L Tris-HCl (pH 7.0), 600 mmol/L sucrose, 150 mmol/L KF, 15 mmol/L EDTA, 50 mmol/L β-mercaptoethanol, 0.5 mmol/L Phenylmethylsulfonyl fluoride (PMSF). The homogenates were centrifuged at 9,200 g for 15 min. at 4°C, and protein content in the supernatant was determined by the method of Bradford [20], using a Bio-

Rad protein assay kit (Bio-Rad, Hertfordshire, U.K.). The GS reaction was initiated by adding 50 μg of protein from the hepatocytes supernatant in 30 μl, to 60 μl of a pre-warmed reaction mixture composed of 50 mmol/L Tris-HCl (pH 7.8), 25 mmol/L NaF, 20 mmol/L EDTA, 10 g/L glycogen and 0.2 mmol/L uridine diphosphate glucose (UDP-[^{14}C]glucose) (American Radio labeled Chemicals, St. Louis, MO). Total GS and GS_I activity were determined in liver homogenates in the presence of 4 mmol/L glucose-6-phosphate (G-6-P) and 0.2 mmol/L G-6-P, respectively. The reaction was performed at 30°C for 15 min and stopped by spotting 75 μL aliquots of the mixture on filter paper squares, immediately washed in 66% (v/v) ethanol for 20 min (three times) and once in acetone for 5 min. The filter papers were dried and counted in a liquid scintillation counter. Enzyme activity was expressed as nmoles UDP-Glucose incorporated into glycogen per mg protein.

2.5. Immunoblot analysis

Hepatocyte homogenates were prepared as described above for GS assay, and 50 μg of protein from the supernatant was boiled in the presence of 25 mmol/L Tris-HCl (pH 6.8), 0.36 mol/L 223 -mercaptoethanol and 10 g/L SDS. Samples were electrophoresed through a 7.5% slab gel in the presence of SDS and electrotransferred to a nitrocellulose membrane, as described in Towbin et al. [21]. The membrane was blocked in TBST [Tris buffered saline pH 7.4 with 0.1% (v/v) Tween 20 (Sigma Chemical, St. Louis, MO)], incubated with polyclonal antibodies for GS (kindly provided by G. Bai, University of Miami, Miami, FL) or GLUT-2 protein (East acres Biologicals, Southbridge, MA) and then reacted with alkaline phosphatase anti-sheep IgG antibody. The filters were washed again with TBST and color stained with 5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium (BCIP/NBT) complex. Rat liver GS was used as a control in the immunoblot assay.

2.6. Statistical analysis

Data are expressed as means ± SEM. Group means were compared by one-way analysis of variance (ANOVA). Means were considered significantly different at $P \leq 0.05$, as determined by Fisher's protected least significant difference method. For comparisons made between treatments within an experimental group (with or without insulin) an unpaired T-test was carried out. In order to equalize variance, when necessary, data were log transformed.

3. Results

Experiment 1: The effect of protein and mRNA synthesis inhibitors on insulin induced stimulation of GS activity and expression was determined in freshly prepared hepatocytes. GS activity is shown in Figure 1A. Insulin significantly

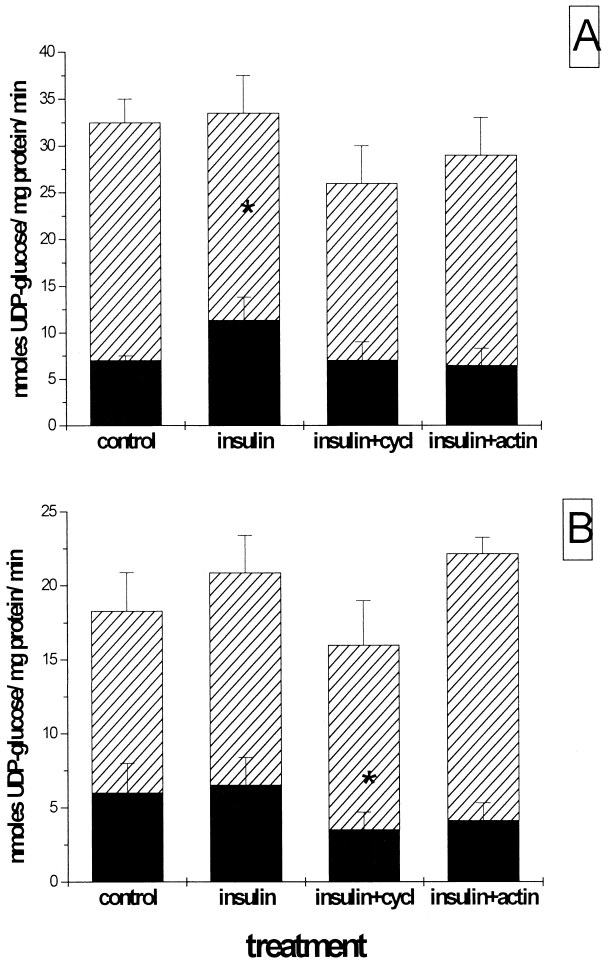

Fig. 1. Glycogen synthase activity in hepatocytes from healthy (Panel A) and diabetic (Panel B) rats following incubations for 45 min with insulin (insulin treatment); with insulin and cycloheximide (insulin + cycl. treatment) or with actinomycin (insulin + actin. treatment). GS_I activity (■) was determined with 0.2 mmol/L G6P and GS_T (▨) in the presence of 4 mmol/L G6P. Results are mean ± SEM (n = 5). * = Significantly different from all other treatments. $P < 0.05$.

increased the synthase I activity (GS_I form) from 7.0 ± 0.5 to 11.3 ± 2.5 nmol UDP-glucose/mg protein/min, but only a small increase in total GS activity was observed when measured in the presence of 4 mmole G-6-P (GS_T form). Cycloheximide and actinomycin abolished the stimulatory effect of insulin. The GS_I activity in the presence of the inhibitors was 7.0 ± 2.0 and 6.4 ± 1.9 nmol UDP-glucose/mg protein/min, respectively. A distinct trend towards lower total GS_T activity in the presence of both cycloheximide and actinomycin was observed, but significant differences were not reached. GS protein content in response to these treatments was assayed by immunoblots (Table 2). Insulin significantly increased GS content in hepatocytes in comparison to controls and the incubation of hepatocytes with cycloheximide and actinomycin significantly inhibited the stimulatory effects of insulin. The inhibitory effect was only observed in response to insulin stimulation and not found in control incubations.

Table 2
Glycogen synthase protein content in hepatocytes isolated from healthy and diabetic rats and incubated for 45 min with or without insulin; with insulin and cycloheximide, or with insulin and actinomycin

Treatment	% Control density*	
	Healthy rats	Diabetic rats
Control	100[b]	100[b]
Insulin	120 ± 5[a]	147 ± 14[a]
Insulin + cycloheximide	69 ± 10[c]	72 ± 9[c]
Insulin + actinomycin	89 ± 10[bc]	107.8 ± 12[b]

* Data are presented as % control.
Results are mean ± SEM of five experiments.
a,b,c—Different letters represent significant difference among values within columns $p < 0.05$.

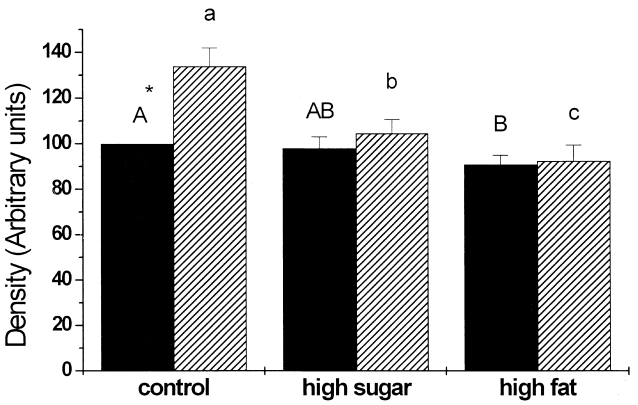

Fig. 2. Immunoblots of Glycogen synthase content in livers of rats fed regular chow diet; a diet rich in simple sugars or a high fat diet. Hepatocytes were incubated for 45 min with (▨) or without (■) insulin (n = 7 rats per group). Data are presented as % control. Results are mean ± SEM. Different letters represent significant differences among diets $p < 0.001$, * represents a significant difference (+) Vs (−) insulin within diet $p < 0.05$.

In Experiment 2, rats were fed experimental diets (Table 1) for a 4 week period and at the termination of the experiment, blood samples were taken and hepatocytes harvested. Similar weight gain was recorded for all three groups of rats. The terminal body weights were 274 ± 3; 271 ± 3; and 272 ± 3 for control, high fat, high carbohydrates diets respectively. Blood glucose and insulin were assayed and rats fed regular chow (control) diet had significantly ($p < 0.05$) lower glucose, 6.62 ± 0.23 mmol/L, in comparison to animals fed the simple sugar (8.03 ± 0.32 mmol/L) or high fat (8.57 ± 0.31 mmol/L) diets. Blood insulin levels in the control rats were 151.5 ± 11.6 pmol/L, significantly lower than the levels found in either rats fed the diet rich in simple sugars (343.2 ± 35.8 pmol/L) or the high fat diet (350.6 ± 47.2 pmol/L).

Interactions between diet and insulin action in glycogen metabolism were assessed by measuring GS activity in hepatocytes from rats fed the experimental diets. Table 3 shows that GS_I activity in hepatocytes from rats fed the control diet was doubled by the addition of insulin from 9.6 ± 1.8 to 19.1 ± 2.8 nmoles UDP-glucose/mg protein/min. GS_I activity in rats fed carbohydrate rich diet was significantly lower than those fed the control diet (6.0 ± 0.4) but was also significantly ($p < 0.001$) stimulated by insulin, but to a lesser extent. GS_I activity in rats fed a fat rich diet was significantly lower than both the control and carbohydrate fed rats, and showed no significant response to insulin. The same trend was observed when GS_T activity was assayed. Total GS activity in control was nearly doubled by the addition of insulin, from 28.5 ± 4.9 to 50.6 ± 5.3 nmoles UDP-glucose/mg protein/min. GS_T from carbohydrate diet fed rats was also significantly stimulated by insulin but to a lesser extent (31.2 ± 6.0 to 40.8 ± 4.0). No significant effect in Total GS activity was observed when hepatocytes of rats fed fat rich diet were incubated with insulin. When fractional activity was calculated, it was noted that the high carbohydrate diet significantly reduced this parameter whereas the high fat diet significantly reduced both fractional activity and total activity. GS protein content in hepatocytes isolated from control rats increased by 40% in the presence of insulin as measured by western blot (Figure 2). In contrast, GS levels in hepatocytes from rats fed the high simple sugar or high fat diet were not affected by insulin. The overall expression of GS protein was control > high simple sugar > high fat with significant differences measured between the control and high fat diets. In the presence of insulin similar results were observed.

GLUT-2 has been identified as the primary glucose transporter in liver cells. GLUT-2 expression was measured in hepatocytes from rats fed various experimental diets. Results show (Figure 3) that in animals fed the control diet,

Table 3
Glycogen synthase activity in hepatocytes isolated from rats fed a control, high simple sugar or high fat diet and incubated for 45 min with or without insulin

	GS_I activity		GS_T activity		Fractional activity	
	− insulin	+ insulin	− insulin	+ insulin	− insulin	+ insulin
Control	9.6 ± 1.8*[a]	19.1 ± 2.8[a]	28.5 ± 4.9*[a]	50.6 ± 5.3[a]	0.33[a]	0.38[a]
High carbohydrate	6.0 ± 0.4*[b]	9.3 ± 1.4[b]	31.2 ± 6.0**[a]	40.8 ± 4.0[a]	0.19[b]	0.22[b]
High fat	3.6 ± 0.4[c]	4.3 ± 0.7[c]	14.8 ± 1.4[b]	16.0 ± 2.1[b]	0.24[b]	0.27[b]

Control (n = 6) other diets (n = 7).
* (−) insulin vs. (+) insulin $p < 0.001$.
** (−) insulin vs. (+) insulin $p < 0.05$.
a,b,c—different letters represent significant difference within columns $p < 0.05$.

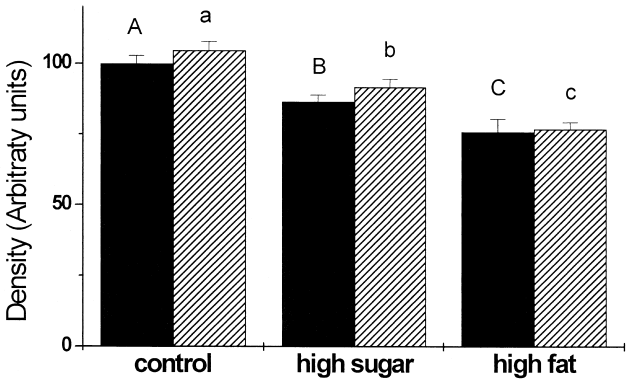

Fig. 3. GLUT-2 content assayed by immunoblot in hepatocytes from rats fed regular chow diet; a high simple sugar diet or a high fat diet. Liver cells were incubated for 45 min with (▨) or without (■) insulin (n = 7 rats per group). Data are presented as % control. Results are Mean ± SEM. Different letters represent significant difference between diets $p < 0.001$.

there was a significantly higher expression of GLUT 2 protein in comparison to rats fed either the high simple sugar or high fat diet. The simple sugar diet also had significantly higher GLUT-2 expression when compared to the high fat diet. Incubation with insulin did not significantly change hepatic GLUT-2 expression.

Experiment 3: GS activity and GS protein content were assayed in hepatocytes from diabetic animals (Figure 1B). GS_I activity was 6.0 ± 2.0 nmoles UDP-glucose/mg protein/min. In comparison to healthy rats GS_I was slightly inhibited in diabetic rats, while GS_T was markedly lower (Figure 1A). Insulin stimulation did not increase GS_I activity in hepatocytes from diabetic rats. The activity of GS_I in hepatocytes of diabetic rats was significantly inhibited by cycloheximide by 50%, while actinomycin also tended to inhibit GS_I activity. No significant effect was observed on GS_T activity, although a trend towards decreased activity was observed in the hepatocytes exposed to cycloheximide. In contrast to the minor effect of insulin on diabetic rats' GS activity (Figure 1B), insulin increased GS protein content in diabetic rats by 47% (Table 2). Cycloheximide markedly inhibited the insulin stimulatory effect on protein content while actinomycin neutralized insulin stimulation of GS protein expression, and protein content was not different from control levels.

4. Discussion

This study examined hepatic GS regulation by insulin, in combination with dietary manipulation and diabetes, both known to affect hepatic glycogen metabolism. Experiment 1, carried out in hepatocytes isolated from healthy rats, showed that GS_I activity was stimulated by insulin (Figure 1A). Cycloheximide and actinomycin both inhibited insulin induced GS_I activity, and reduced it to the level of control hepatocytes indicating that new protein transcription and synthesis are associated with insulin stimulation. (Table 2). GS activity levels correspond well with the results of Western Blots showing increased GS protein in the presence of insulin and lower levels of GS protein in hepatocytes exposed to cyclohexamide and actinomycin. Inhibiting protein synthesis and RNA synthesis appears to directly decrease GS levels, but also can modify GS activity via inhibition of regulatory proteins, phosphorylase and kinase [22].

The effects of dietary manipulation on hepatic GS were also investigated in this study. Nutritional models of impaired glucose metabolism were chosen. It has been documented that consumption of high fat or high simple sugar diets can lead to insulin resistance in rats [23–25]. Kragen et al. [15] reported that high fat feeding induced insulin resistance initially in rat liver and adipose tissues, followed by impaired glucose metabolism skeletal tissues. We found that rats fed high simple carbohydrate or high fat diets had significantly higher fasting plasma insulin and glucose levels compared to control animals, characteristic symptoms of insulin resistance. The nutritional state is a factor known to affect the hepatic glycogen levels and the glycogenolytic state, as seen in livers from fasted, fed and fasted-refed rats [26,27]. In comparison to control animals, hepatocytes from rats fed high simple sugar or high fat diets had significantly decreased GS_I activity. This was true for both basal GS activity and activity stimulated by insulin. This finding confirms reports by others who observed a significant inhibition of glucose incorporation into glycogen in rats fed a high fat diet [16]. Western blots of GS protein (Figure 2) indicate that the control rats responded to insulin stimulation while the high fat and high simple sugar diets had a minimal response. These results support the findings that high fat and high simple sugar diets lead to insulin resistance as observed in lower rates of both GS activity and expression than in control animals.

The liver plays a key role in glucose homeostasis, therefore the effects of dietary manipulations on glucose transporter expression is of great interest. The rate of hepatic glucose uptake by GLUT-2 increases in parallel with the rise in blood glucose over the physiological concentration range. The expression of the transporter is regulated by glucose and different hormones [28]. In male Wistar rats it was found that intravenous nutrition induced insulin resistance and reduced liver GLUT-2 protein and mRNA levels. The authors interpretation of the results suggest that the route of nutrient delivery influences their utilization by the liver [29]. Indeed, we also found that nutritional manipulation affected GLUT-2 expression (Figure 3). Simple carbohydrate and fat rich diets significantly reduced the expression of GLUT-2 in hepatocytes. However, short term exposure to insulin did not influence GLUT-2 levels in control or experimental animals. This result corresponds well with reports that GLUT-2 is not acutely controlled by insulin [1].

Hepatic insulin resistance is a major defect of non insulin dependent diabetes mellitus. It has been previously demon-

strated that glycogen deposition from glucose is impaired in diabetic animals [1,30]. Glycogen deposition was also impaired in diabetic rats where the activation of glycogen synthase is either impaired or absent, in proportion to the severity of insulin deficiency [31]. In this study, Total GS activity in hepatocytes from diabetic rats was markedly lower than in hepatocytes from healthy rats while a trend towards lower GS_I activity was also observed. In the presence of insulin, GS_I activity was nearly doubled by the addition of insulin in healthy rat hepatocytes, an effect that did not occur in hepatocytes from diabetic rats. These results partially correspond with the results of Rao et al. [10] who found significantly lower GS_I activity in STZ induced diabetic rats but no change in total glycogen synthase activity. In this study, GS_I activity was not significantly changed in diabetic animals however, total activity was decreased indicating a defect in GS activity or expression in the diabetic state. It is well known that glycogen synthase dephosphorylation in the liver is lowered in insulin deficient animals. It has been suggested that the decrease in synthase phosphatase, which involves the glycogen bound form of the enzyme, could be the basis for the defect in the deposition of glycogen from glucose in the liver of diabetic animals [32], as well as in other tissues [33]. Indeed, our results demonstrate that GS activity in diabetic animals was minimally affected by the addition of insulin to the incubation medium (Figure 1B), namely these cells were not sensitive to regulation by insulin in comparison to the healthy (control) animals (Figure 1A). Insulin increased GS protein content in cells from diabetic animals (Table 2) further supporting the hypothesis that a defect in synthase dephosphorylation limits activity in the Type I diabetes, rather than depleted GS levels (Figure 1B).

These parameters have been previously analyzed in our laboratory in response to starvation and refeeding cycles in rats. GS activity and protein expression were found to be responsive to the change in the animals' diet, as well as the insulin effect [26]. The results presented in this study further confirm the important role of glycogen synthase activity and its regulation by various diet and hormonal factors.

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