Glycogen Levels and Energy Status of the Liver of Fasting Rats with Diabetes Types 1 and 2

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ABSTRACT

Glycogen levels and the energy status of livers from fasting rats with diabetes types 1 and 2 were measured. After a 24 h fast, the hepatic glycogen levels of rats with diabetes1 and diabetes2 were, 18.7 and 2.6 times higher, respectively, than those of livers from the normal rats. In diabetes1 rats, the glycogen levels decreased when the fasting period was extended to 48 and 72 h. The opposite occurred with the control and diabetes2 rats. Consistently, glucose release by the perfused livers from diabetes1 rats was considerably higher during at least 60 minutes after initiating perfusion. The hepatic ATP content of diabetes1 rats was similar to that of the control rats; in diabetes2 rats, the hepatic ATP content was increased. It could be concluded that regulation of glycogen deposition and degradation in rats with diabetes1 differed markedly from that of rats with diabetes2 which, in turn, behaved similarly to normal healthy rats.

Key words: Diabetes type 1, diabetes type 2, rats, hepatic glycogen, hepatic energy status

INTRODUCTION

In spite of decades of experimental efforts, there are many aspects of the hepatic metabolism of diabetic animals that have not yet be satisfactorily clarified. Contradictory observations are frequently found in the specialized literature. For example, earlier experiments with isolated hepatocytes of alloxan diabetic rats (diabetes type 1) have led to the conclusion that hepatic gluconeogenesis from alanine, pyruvate and fructose is considerably increased in this preparation (Wagle et al., 1975). Experiments with the isolated perfused rat liver, however, concluded that gluconeogenesis from lactate, glycerol and sorbitol is not affected by alloxan and streptozotocin induced diabetes, whereas gluconeogenesis from alanine is absent in these animals (Ferraz et al., 1997). Kleckner et al. (1987), on the other hand, have found diminished rates of gluconeogenesis from lactate in perfused livers from alloxan diabetic rats (diabetes type 1). A common characteristic of all these experiments is that livers from rats with diabetes type 1 present high rates of basal glucose release, i.e., before the introduction of the gluconeogenic substrates. This could be due to glycogenolysis or gluconeogenesis from endogenous substrates. Actually, it has been reported that the glycogen levels of rats with diabetes 1 after a 24 h fast are much higher than those in normal healthy rats (Gannon and Nuttall, 1997), so that the elevated basal rates of glucose release observed by several authors could be the result of glycogenolysis.

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The present work attempted to investigate how the hepatic glycogen levels of diabetic rats behave during various fasting periods. It is known that in healthy rats, the hepatic glycogen levels tend to increase again after a 48 h fast (Sasse, 1975; Bois-Joyeux et al., 1987), a phenomenon which reflects most probably increased gluconeogenesis from endogenous substrates such as glutamine (Mouterde et al., 1992). In this respect, the behavior of diabetic rats is not known and an investigation of this question could be important for two main reasons. Firstly, it would reveal hitherto unknown aspects of the metabolic behavior of diabetic animals, especially if the study is extended to rats with diabetes type 2 in addition to rats with diabetes type 1. Second, it should also produce basic information which can be used for planning future experiments in which gluconeogenesis from several substrates in fasting diabetic rats will be investigated. For this reason, in the present work, the hepatic glycogen levels of normal rats and rats with diabetes types 1 and 2 were followed over a period of 72 h after food deprivation. In addition, other parameters were measured such as liver weight and the hepatic energy status.

MATERIAL AND METHODS

Materials
All the enzymes and coenzymes used in the enzymatic assays were purchased from Sigma Chemical Co. (St Louis, USA). All the standard chemicals were from the best available grade (98-99.8 % purity).

Animals and diabetes induction
Male Wistar rats weighing 220–250 g fed with a standard laboratory diet (Nuvilab®) were used in all perfusion experiments. Diabetes type 1 (diabetes1) was induced by injecting adult rats with streptozotocin (Gannon and Nuttall, 1997). Streptozotocin was dissolved in citrate buffer (10 mM; pH 4.5) and a single dose of 50 mg/kg was injected intraperitoneally. After 12 days, blood glucose of fed rats was measured and animals presenting glycemic levels equal or above 16 mM in the fed state were selected for the experiments. Control rats were injected intraperitoneally with citrate buffer (10 mM, pH 4.5).
Diabetes mellitus type 2 (diabetes2) was induced

as described by Portha et al. (1974). Male newborn (2 days old) Wistar rats were injected intraperitoneally with streptozotocin (150 mg/ kg) dissolved in citrate buffer (10 mM; pH 4.5). Seven weeks later, diabetes was confirmed by urine glucose levels, 24 h urinary volume and water intake. The protocol for these experiments was approved by the Ethics Committee for Animal Experimentation of the University of Maringá.

Liver perfusion
Hemoglobin-free, non-recir-culating perfusion was done according to the technique described elsewhere (Scholz and Bücher, 1965; Kelmer-Bracht et al., 2002). For the surgical procedure, the rats were anesthetized by intraperitoneal injection of sodium thiopental (50 mg/kg). After cannulation of the portal and cava veins the liver was positioned in a plexiglass chamber. The flow was maintained constant by a peristaltic pump (Minipuls 3, Gilson, France) and was adjusted between 30 and 35 ml min^{-1}, depending on the liver weight. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4), saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment at 36°C. The composition of the Krebs/Henseleit-bicarbonate buffer is the following: 115 mM NaCl, 25 mM NaHCO$_3$, 5.8 mM KCl, 1.2 mM Na$_2$SO$_4$, 1.18 mM MgCl$_2$, 1.2 mM NaH$_2$PO$_4$ and 2.5 mM CaCl$_2$. Glucose in the outflowing perfusate was measured enzymatically (Berg-meyer and Bernt, 1974).

Assays
Glycogen was determined in freshly isolated livers from anesthetized rats (sodium thiopental, 50 mg/kg). Portions of approximately 2 g were freeze-clamped with liquid nitrogen. This sample was homogenized and extracted with 8 ml of 6% HClO$_4$. The supernatant was neutralized with 5 N K$_2$CO$_3$ and used for the enzymatic glycogen assay (Kepler and Decker, 1974).
For the measurement of the cellular adenine nucleotide contents, livers were freeze-clamped in liquid nitrogen, homogenized and extracted with perchloric acid. The neutralized extracts were used for the enzymatic assay of ATP (Lamprecht and Trautschold, 1974), ADP and AMP (Jaworek et al., 1974).
Plasma concentrations of glucose, urea and ammonia were assayed by collecting blood from

the vena cava. Blood was collected by means of a syringe containing heparin after a surgical incision into the abdominal cavity of anaesthetized rats (sodium thiopental, 50 mg/kg). After sedimenting the blood cells in a refrigerated centrifuge (10 minutes, 7000 rpm), plasma was used for the enzymatic assays of urea (Gutmann and Bergmeyer, 1974), ammonia (Kearney and Kun, 1974) and glucose (Bergmeyer and Bernt, 1974).

Treatment of data
Statistical analysis of the data was done by means of the Statistica^{TM} program (Statsoft$^®$, 1998). Mono- or two-way variance analysis was applied according to context with post-hoc testing; p < 0.05 was adopted as a criterion of significance.

RESULTS AND DISCUSSION

Table 1 shows some characteristics of the plasma of the rats with diabetes types 1 and 2 in the fasted state. After a fasting period of 24 h the plasma glucose concentrations of both rats with diabetes1 and 2 were 57.3% and 23.1%, respectively, above the normal values. These values were smaller than those ones normally found in fed diabetic rats (Portha et al., 1974; Gannon and Nuttall, 1997). The ammonia and urea concentrations of the rats with diabetes2 were normal, but rats with diabetes1 presented considerably higher urea (+401.5%) and ammonia (+90.9%) concentrations. These higher blood urea and ammonia concentrations in rats with diabetes1 are probably reflecting the higher rates of amino acid degradation (Squires et al., 1997), which also increase the urinary urea excretion (Kim et al., 2005). However, the increased urea excretion was combined with a much higher urinary volume and lower urinary urea concentrations (Kim et al., 2005). The high blood urea concentrations are, thus, the consequence of both higher amino acid catabolism and impaired renal function. Investigations about the urea cycle in the liver of diabetes1 rats would be highly desirable because this tissue is the main source of urea. It is interesting to note that rats with diabetes2 did not present such visible alterations in nitrogen catabolism.
Fig. 1 illustrates the changes in the hepatic glycogen contents and the relative liver weights as a function of the fasting time. Rats with diabetes1 presented higher liver weights per body weight (g liver per 100 gram body weight) after a 24 h fast. The difference vanished, however, when the fasting period was increased. For rats with diabetes2, the relative liver weight was similar to that of the control rats, irrespective of the fasting period. The higher liver weights of diabetes1 rats after a 24 h fast could be caused, partly at least, by their high glycogen contents because of the pronounced hygroscopic properties of this polysaccharide. As shown in Fig. 1, rats with type 1 diabetes contained high levels of hepatic glycogen after a 24 h fast, with a diabetes1/control ratio equal to 18.7. It should be noted that the relatively high levels of hepatic glycogen in 24 h fasted diabetes1 rats, 55.8 µmol g^{-1}, were actually much lower than the maximal levels normally attained by the normal fed rats which may reach values as high as 330 µmol g^{-1} (Bazotte et al., 1990). Rats with diabetes type 2 also presented higher hepatic glycogen levels, namely 8.2 µmol g^{-1}, but the diabetes2/control ratio was only 2.76. Prolongation of the fasting period produced decreases in the glycogen levels of rats with diabetes1 and increases in the glycogen levels of rats with diabetes2 as well as in control rats. At 72 h, all the groups presented similar hepatic glycogen concentrations. These observations mean that no net glycogenesis took place in diabetes1 rats during the period between 24 and 72 h fast. Net glycogen synthesis, however, must have occurred in the control and diabetes2 rats during the same period. In the absence of exogenous sources, this net glycogen accumulation must have occurred at the expense of gluconeogenesis, which in turn, depended exclusively on endogenous sources.
Since diabetes1 rats presented relatively high glycogen levels, glucose release by the perfused livers of such animals should be relatively pronounced if no exogenous gluconeogenic substrates were added to the perfusion fluid. This hypothesis was tested by perfusing the livers from both control and diabetes1 animals with substrate-free perfusion fluid. Sampling of the outflowing perfusate for glucose assay was initiated immediately after connecting the liver to the perfusion apparatus.
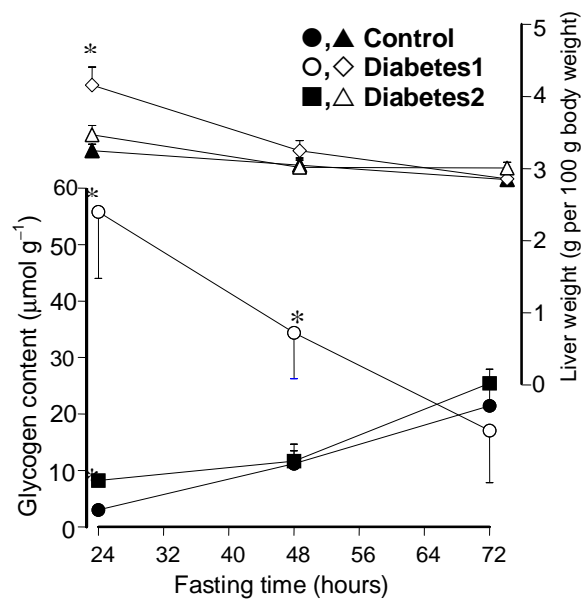
Figure 1 - Evolution of liver weight and glycogen content as a function of the fasting time in control rats and rats with diabetes1 and 2**.** Statistically significant differences between diabetes1 and control or diabetes2 and control are indicated by asterisks ($p < 0.05$). Bars represent mean standard errors of a minimum of 3 and a maximum of 17 determinations
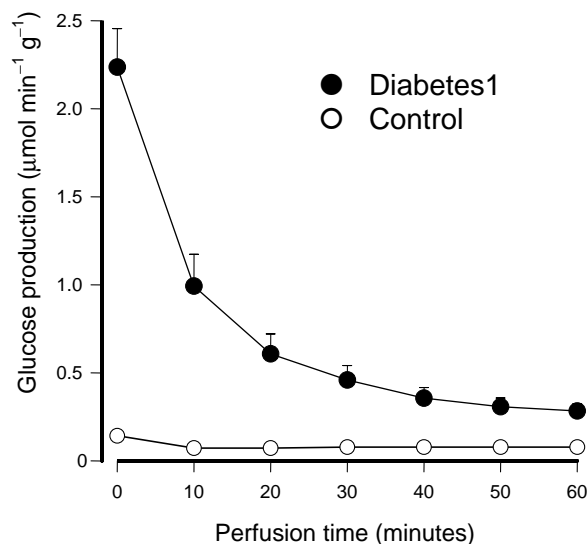
Figure 2 - Rates of glucose production by rat livers as a function of time after connecting the liver to the perfusion apparatus. Livers were perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4). Sampling of the outflowing perfusate was initiated immediately after connecting the liver to the perfusion apparatus. Glucose in the outflowing perfusate was measured enzymatically. Data are means ± SEM of 6 (control) and 12 (diabetes1) liver perfusion experi-ments

Table 1 - Plasma concentrations of glucose, ammonia and urea in 24-h fasted control and diabetic rats. Blood was collected and analyzed enzymatically. Data are means ± SEM. Asterisks are used to indicate values differing statistically from the corresponding controls according to the Student-Newman-Keuls test applied after variance analysis ($p < 0.05$).

Compound	Control	Diabetes1	Diabetes2
mM			
Glucose	4.80±0.14 (n=5)	7.55±0.47* (n=18)	5.91±0.25* (n=9)
Ammonia	0.11±0.01 (n=8)	0.21±0.03* (n=11)	0.14±0.01 (n=7)
Urea	4.83±0.26 (n=8)	24.22±1.68* (n=11)	4.74±0.42 (n=7)

Table 2 - Hepatic contents of adenine nucleotides in 24-h fasted control and diabetic rats. The liver freeze-clamped in liquid nitrogen, extracted with cold perchloric acid and the adenine nucleotides were determined enzymatically. Data are means ± SEM. Asterisks are used to indicate values of the diabetes1 or diabetes2 conditions differing statistically from the corresponding controls according to the Student-Newman-Keuls test applied after variance analysis ($p < 0.05$).

Animal condition	ATP	ADP	AMP	Total nucleotides
	µmol (gram liver wet weight)$^{-1}$			
Control (n=13)	1.42±0.17	1.16±0.05	0.35±0.05	2.93±0.11
Diabetes1 (n=4)	1.29±0.12	0.90±0.03*	0.32±0.07	2.51±0.10*
Diabetes2 (n=9)	2.16±0.08*	1.11±0.02	0.25±0.02	3.52±0.08*

The results are shown in Fig. 2. Glucose release from the control livers was small and almost constant over the 60 minutes perfusion time. Livers from diabetes1 animals, however, showed initially very high rates of glucose release, with a diabetes1/control ratio equal to 15.6 at time zero. These high rates diminished progressively during the subsequent time and the diabetes1/control ratio was reduced to 3.59 at 60 minutes perfusion time. This decrease represents most probably the progressive diminution of the glycogen stores in consequence of glycogenolysis.

Table 2 shows the tissue contents in adenine nucleotides of livers from control, diabetes1 and diabetes2 animals after a fasting period of 24 h. Diabetes1 rats presented a small tendency toward smaller ATP levels, but only the ADP levels were statistically smaller than those found in control livers. As a consequence, however, the sum of total nucleotides (ATP+ADP+AMP) was also somewhat smaller in diabetes1 rats. Diabetes2 rats, on the other hand, presented significantly higher ATP levels with no significant changes in the AMP and ADP levels. In consequence, these rats also presented a higher content in total adenine nucleotides. These results did not reveal any significant ATP depletion in diabetic rats after a

24 h fast. The reasons for the higher ATP levels in the livers from diabetes2 rats cannot be inferred from the present data, but the phenomenon is clearly worth of more specific investigations.

It is quite apparent that investigations concerning gluconeogenesis in livers from diabetes1 rats must take into account the relatively high glycogen levels which are unavoidably associated to glycogenolysis in the perfused liver, with high and declining rates of glucose release. For distinguishing gluconeogenesis from glycogenolysis under these conditions, it will be indispensable to use radioactive precursors, which allow to measure newly formed glucose in a specific manner. Alternatively, gluconeogenesis can also be evaluated if the glycogen levels are simultaneously measured so that the appropriate corrections can be done. Simple subtraction of the very high basal rates after infusion of a gluconeogenic substrate (Ferraz et al., 1997; Akimoto et al., 2000) is a highly risky procedure which is likely to lead to erroneous evaluations of the true gluconeogenic activities.

The relatively high hepatic glycogen levels of 24 h-fasted rats with diabetes type 1 are probably the consequence of the high glycogen synthase to glycogen phosphorylase activity ratio, as reported

by several workers (Akatsuka et al., 1983; Rao et al., 1995; Gannon and Nuttall, 1997). No such data are available about rats with diabetes type 2. However, taking into account that these rats present considerably lower glycogen levels when compared to rats with diabetes type 1, it is conceivable that they also present lower glycogen synthase/glycogen phosphorylase levels. On the other hand, the progressive decrease of the hepatic glycogen levels in diabetes1 rats when the fasting period was prolonged to 72 h could be reflecting also a progressive decrease in the glycogen synthase/ glycogen phosphorylase ratio, the opposite being more likely in healthy (control) and diabetes2 rats. The phenomenon of increased glycogen accumulation after a prolonged fasting period, common to healthy and diabetes2 rats, but contrasting with diabetes1 rats, is poorly under-stood. However, since glycogen synthesis under starvation depends essentially on the amino acid availability resulting from protein degradation, one can expect that amino acids are playing a regulatory role. Such a role has been attributed to glutamine based on specific experiments with hepatocytes isolated from rats after different fasting periods (Mouterde et al., 1992). In hepatocytes isolated from 72 h fasted rats, glutamine is more effective in stimulating glycogen synthase than after a 24 h fast. At the same time, the capacity of glutamine in stimulating glycogen phosphorylase, clearly present after a 24 h fast, is practically absent after a 72 h fasting period.

In conclusion, regulation of glycogen deposition and degradation during the prolonged fasting periods in rats with diabetes1 differs markedly from that of rats with diabetes2 which, in turn, behave similarly to normal healthy rats. There is little information available about the mechanisms underlying these differences, but data available about the influence of glutamine on glycogen synthase activation during prolonged starvation (Mouterde et al., 1992) suggest a crucial role for the amino acids resulting from proteolysis.

RESUMO

Teores de glicogênio e os estados energéticos de fígados de ratos com diabete dos tipos 1 e 2 foram medidos. Após um jejum de 24 horas os teores de glicogênio de ratos com diabete1 e diabete2 foram, respectivamente 18,7 e 2,6 vezes superiores àqueles de fígados de animais controle. Em ratos com diabete1 o conteúdo de glicogênio diminuiu quando o período de jejum foi prolongado para 48 e 72 horas. O oposto ocorreu em ratos controle e ratos com diabete2. Consistentemente, a liberação de glicose por fígados em perfusão isolada obtidos de ratos com diabete1 foi consideravelmente maior durante ao menos 60 minutos após o início da perfusão. O conteúdo hepático de ATP de ratos com diabete1 foi similar àquele de ratos controle; em ratos com diabete2 o conteúdo hepático de ATP foi maior. Pode-se concluir que a regulação da deposição e degradação do glicogênio em ratos com diabete1 difere marcadamente daquela de ratos com diabete2, os quais, por seu turno, comportam-se similarmente a ratos normais e saudáveis.

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