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Evidence that GLUT-2 mRNA and protein concentrations are decreased by hyperinsulinaemia and increased by hyperglycaemia in liver of diabetic rats

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GLUT-2, glucokinase (GK) and phosphoenolpyruvate carboxykinase (PEPCK) mRNA expression was studied in the liver of chronically catheterized diabetic rats during the 3 days after an intravenous injection of 65 mg of streptozotocin (STZ)/kg. At 6 h after the STZ injection, portal plasma insulin levels were 270 ± 32 μ-units/ml and blood glucose was 1.4 ± 0.4 mmol/l, owing to pancreatic β-cell destruction. GLUT-2 and PEPCK mRNA concentrations were rapidly and dramatically decreased ($> 90\%$), whereas GK mRNA was increased. After 30 h, plasma insulin concentrations were lower than 5 μ-units/ml and blood glucose was > 20 mmol/l. GLUT-2 and PEPCK mRNA concentrations increased 2-fold and GK mRNA disappeared progressively. In order to assess the relative roles of hyperglycaemia and insulinopenia, blood glucose was clamped at 6.4 ± 0.5 mmol/l from 18 to 72 h after STZ injection by phlorizin infusion (0.5–2 g/day per kg) or at 6.6 ± 0.3 mmol/l from 18 to 48 h after STZ injection by insulin infusion (0.25 unit/min per kg). GLUT-2 mRNA concentrations were 50% lower in phlorizin-infused than in untreated diabetic rats. The low levels of GK mRNA and the high levels of PEPCK mRNA were unaffected by normalization of hyperglycaemia in phlorizin-infused diabetic rats. In insulin-infused rats (portal plasma insulin levels of 40 μ-units/ml) GLUT-2 mRNA levels were 25% of those in untreated diabetic rats, and they increased rapidly 6 h after insulin infusion was stopped. Liver GLUT-2 protein concentration showed similar changes in response to STZ injection and to phlorizin or insulin treatment, but after a delay of several hours. From this work we conclude that GLUT-2 gene expression is dramatically and rapidly (< 6 h) decreased by portal hyperinsulinaemia and increased by hyperglycaemia.

INTRODUCTION

Glucose homoeostasis is a tightly regulated phenomenon that involves glucose uptake by peripheral tissues and glucose storage by, or production from, the liver. The transport of glucose into the cell represents the first step at which glucose utilization is controlled in muscles and adipose tissue (Simpson & Cushman, 1986), whereas in the liver glucose transport is not thought to be rate-limiting for glucose metabolism in normal conditions. In muscle and fat, glucose transport is acutely stimulated by insulin (Simpson & Cushman, 1986), but in the liver glucose transport is not sensitive to insulin (Williams *et al.*, 1968; Bauer & Heldt, 1977). More recently, it has been shown that glucose uptake and release required a family of membrane facilitated-diffusion glucose transporters which are expressed in a tissue-specific manner (Burant *et al.*, 1991). In muscle and fat, GLUT-4 is the main isoform of glucose transporters (Burant *et al.*, 1991). In adipose tissue the concentrations of GLUT-4 protein and mRNA are markedly decreased after 2–3 weeks of diabetes, and they are restored by insulin therapy (Berger *et al.*, 1989; Garvey *et al.*, 1989; Sivitz *et al.*, 1989), whereas in skeletal muscle the concentrations of GLUT-4 protein and mRNA are marginally altered (Garvey *et al.*, 1989; Kahn *et al.*, 1989; Bourey *et al.*, 1990). In liver, GLUT-2 is the main isoform of glucose transporters (Thorens *et al.*, 1988). Much less information is available concerning the expression of GLUT-2 in liver of diabetic rats, and the results are somewhat contradictory. It has been reported that the concentrations of GLUT-2 protein and mRNA were increased 1.6–2-fold after 2–3 weeks of diabetes in rats (Oka *et al.*, 1990; Yamamoto *et al.*, 1991) and that insulin therapy for 5 days restored the concentrations of GLUT-2 protein and mRNA to the levels of non-diabetic rats (Oka *et al.*, 1990). In contrast, GLUT-2 protein and mRNA were marginally affected after 2–3 weeks of diabetes and were not significantly modified by insulin therapy for 1 week (Thorens *et al.*, 1990). The aim of the present study was to investigate the early effect of streptozotocin (STZ)-induced diabetes on GLUT-2 mRNA in liver and to determine the relative role of hyperglycaemia and hypoinsulinaemia in these changes. The expression of two enzymes involved in gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32), and glycolysis, glucokinase (GK; EC 2.7.1.1), was studied in parallel.

EXPERIMENTAL

Animals

Male Wistar rats (250 g) bred in our laboratory were housed at 24 °C with light from 07:00 to 19:00 h. They had free access to water and chow pellets (65% carbohydrate, 11% fat, 24% protein; % of energy). The studies were performed on conscious rats 24 h after implantation of catheters into the two jugular veins under pentobarbital anaesthesia (50 mg/kg body wt). Immediately after surgery, rats were randomly distributed into four different groups and housed in individual cages.

Group 1. This group of rats had free access to food and water. The rats were killed at 08:00 h. This group was termed as 'fed' control rats.

Group 2. This group of rats was injected with STZ (Sigma, St. Louis, MO, U.S.A.) through the left jugular vein, at a dose of 65 mg/kg body wt. Rats had free access to food and water and were killed 6, 18, 30, 48 or 72 h after the STZ injection. This group was termed 'diabetic' rats.

Group 3. A group of diabetic rats was infused intravenously with phlorizin (Sigma) at a variable rate: 0.5–2 g/day per kg

Abbreviations used: STZ, streptozotocin; PEPCK, phosphoenolpyruvate carboxykinase; GK, glucokinase.

Table 1. Changes in portal blood glucose, plasma insulin and plasma glucagon concentrations after injection of STZ (65 mg/kg, intravenous)

Values are means ± S.E.M. from 4–6 animals in each group. Some diabetic rats were infused with either phlorizin (STZ + PHLO) from 18 to 72 h, or insulin (STZ + INS) from 18 to 48 h, and were compared with their respective diabetic rats (STZ 72 h and STZ 48 h). Some insulin-infused diabetic rats had their insulin infusion stopped after 48 h. They were studied 6 and 30 h after insulin withdrawal. *Difference significantly different ($P < 0.05$) from control values. †Difference significantly different ($P < 0.05$) from STZ 72 h. ‡Difference significantly different ($P < 0.05$) from STZ 48 h. §Difference significantly different ($P < 0.05$) from STZ + INS.

		Time after STZ					STZ + PHLO	STZ + INS	Time after insulin withdrawal	
	Controls	6 h	18 h	30 h	48 h	72 h	72 h	48 h	6 h	30 h
Portal blood glucose (mmol/l)	5.1 ± 0.4	1.4 ± 0.4*	4.4 ± 0.5	17.2 ± 1.9*	21.3 ± 1.6*	26.9 ± 1.5*	6.4 ± 0.5†	6.6 ± 0.3‡	18.1 ± 1.8§	22.8 ± 1.9§
Portal plasma insulin (μ-units/ml)	38 ± 5	270 ± 32*	45 ± 6	7 ± 2*	< 5*	< 5*	< 5	40 ± 8‡	< 5§	< 5§
Portal plasma glucagon (ng/ml)	210 ± 35	225 ± 38	410 ± 48*	315 ± 27*	302 ± 46	215 ± 19	280 ± 25	220 ± 25	275 ± 27	335 ± 42§

from the 18 h after STZ injection to 72 h, to maintain their blood glucose level in the normal range. They had free access to food and water during the experimental period. This group was termed 'phlorizin-treated diabetic' rats.

Group 4. A group of STZ-diabetic rats was infused intravenously with insulin (Actrapid; Novo, Copenhagen, Denmark) at a constant rate, 0.25 unit/h per kg, from 18 h after STZ injection to 48 h, to maintain their blood glucose level in the normal range. They had free access to food and water during the experimental period. This group was termed 'Insulin-treated diabetic' rats. In some of these insulin-treated diabetic rats, insulin infusion was stopped after 48 h. The rats were killed 6 or 30 h after insulin withdrawal.

Monitoring of blood glucose and plasma insulin and glucagon levels

Blood glucose and plasma insulin were monitored daily throughout the experimental period in untreated, phlorizin-treated and insulin-treated diabetic rats. Blood glucose was routinely monitored by the glucose oxidase method with a Beckman glucose analyser (Beckman BGA2). Plasma insulin and glucagon concentrations were determined by radioimmunoassay as described previously (Girard *et al.*, 1973).

Killing of animals and tissue sampling

Rats were anaesthetized by an intraperitoneal injection of pentobarbital (60 mg/kg body wt.). Blood was sampled from the portal vein and collected in the presence of 2000 units of aprotinin/ml of blood and EDTA (0.01 M). Blood was immediately centrifuged at 4 °C and the plasma was frozen at −20 °C until insulin, glucagon and glucose determinations. After blood sampling, the liver was rapidly removed, rinsed out in 0.9% NaCl, wiped and frozen at −80 °C.

Quantification of GLUT-2, GK and PEPCK mRNAs

Total RNAs were isolated from liver by the guanidine thiocyanate method (Chomczynski & Sacchi, 1987). The concentration of RNA was determined by the A_{260}, and the RNA solutions were stored at −80 °C until use. All samples had an A_{260}/A_{280} ratio of about 2.0. For Northern-blot analyses, total RNAs (20 μg) were denatured in a solution containing 2.2 mM-formaldehyde and 50% (v/v) formamide by heating at 95 °C for 2 min, size-fractionated by electrophoresis and transferred to a nylon membrane. The GLUT-2 cDNA probe (Thorens *et al.*, 1988), the PEPCK cDNA probe (Yoo-Warren *et al.*, 1981) and the GK cDNA probe (Iynedjian *et al.*, 1987) were gifts from Dr. Bernard Thorens, Dr. Richard Hanson and Dr. Patrick Iynedjian. Probes were labelled with [^{32}P]dCTP with the Multiprime labelling system kit (Amersham International). Hybridizations were performed in solutions containing 42% deionized formamide, 7.5% dextran sulphate, 8 × Denhardt's solution, 40 mM-Tris/HCl, pH 7.5, and 1% SDS, at 42 °C overnight. The membranes were washed twice for 30 min each, with 2 × SSC (1 × SSC = 0.15 M-NaCl/15 mM-trisodium citrate, pH 7.0)/0.1% SDS at 42 °C and twice for 30 min each with 0.1 × SSC/0.1% SDS at 55 °C and exposed for 4–72 h at −80 °C with intensifying screens. Quantification was performed by scanning densitometry.

The Northern blots were hybridized with an oligonucleotide probe representing the sequence 1047–1070 of rat 18 S rRNA (Chan *et al.*, 1984) labelled with [^{32}P]ATP to verify the integrity of RNA and that each lane was loaded with the same amount of total RNA.

Quantification of GLUT-2 by immunoblotting

Liver was homogenized with a motor-driven Teflon pestle at 4 °C in 0.3 M-sucrose/3 mM-dithiothreitol/0.26 unit of aprotinin (Sigma)/ml, 0.1 mM-phenylmethanesulphonyl fluoride at pH 7.4 (1:10, w/v). Liver membranes were prepared as described by Thorens *et al.* (1990). Membranes were resuspended in Laemmli (1970) buffer containing 5% SDS. Samples (100 μg) of membrane protein were resolved by SDS/PAGE (10% gels) and transferred to nitrocellulose filters (Thorens *et al.*, 1990). Equivalent loading and transfer of proteins from all lanes of the gel was verified by Ponceau Red staining of the nitrocellulose filters and Coomassie Blue staining of the gels. Protein was blotted with a polyclonal antiserum prepared against GLUT-2 (gift from Dr. B. Thorens, University of Lausanne, Switzerland) followed by ^{125}I-Protein A (Amersham International, Amersham, Bucks., U.K.). Protein concentration was determined by the Bio-Rad (Munich, Germany) assay with BSA as standard.

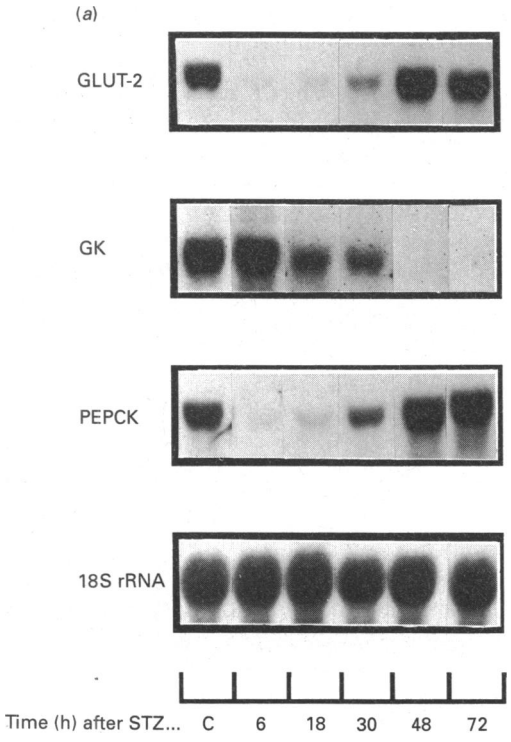
Statistical analysis

Results are expressed as means ± S.E.M. Statistical analysis was performed by Student's *t* test for unpaired data.

RESULTS

Time course of changes in blood glucose and plasma insulin and glucagon concentrations

Fed non-diabetic rats were used as controls. Blood glucose and



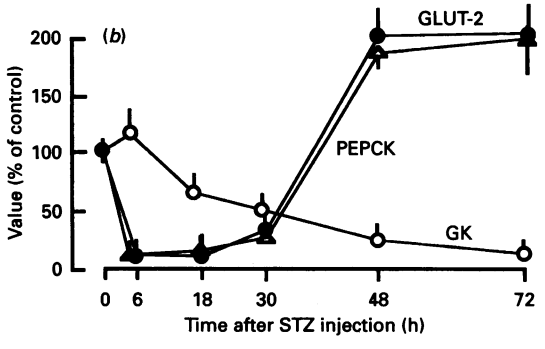


Fig. 1. Time course of changes in liver GLUT-2, GK and PEPCK mRNAs in rats injected with STZ

(*a*) A representative autoradiograph of Northern blots of liver GLUT-2, GK and PEPCK mRNA is presented. (6 controls) (*b*) mRNA was quantified by scaning densitometry of Northern blots. Results are expressed as means ± S.E.M. of 4–6 rats in each group. The values in non-diabetic rats were taken as 100%.

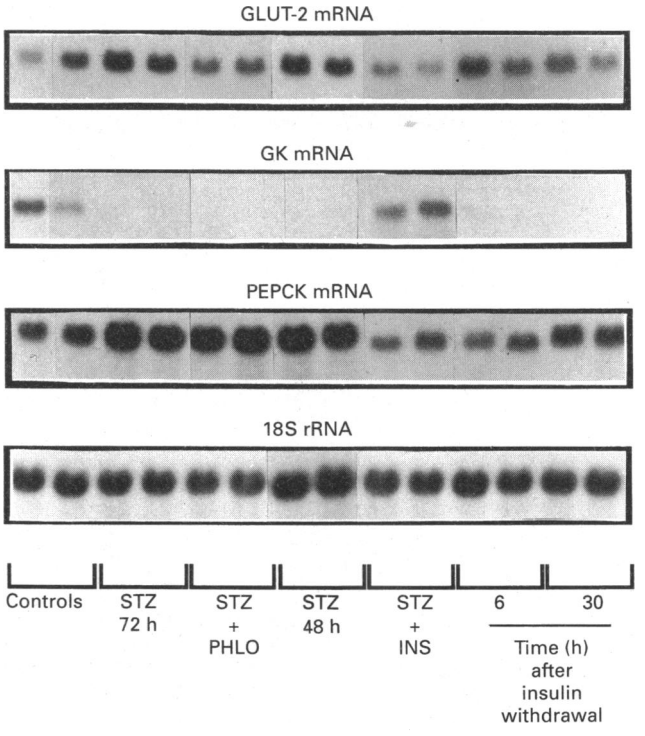


Fig. 2. Representative autoradiograph of Northern blots of liver GLUT-2, GK and PEPCK mRNA in non-diabetic and diabetic rats

Some diabetic rats were infused either with phlorizin (STZ + PHLO) from 18 to 72 h or with insulin (STZ + INS) from 18 to 48 h and were compared with the corresponding diabetic rats (STZ 72 h and STZ 48 h). Some insulin-infused diabetic rats had their insulin infusion stopped after 48 h. They were studied 6 and 30 h after insulin withdrawal. Two Northern blots for each condition are presented.

plasma insulin and glucagon concentrations in control rats were considered as the initial value during the time-course experiments.

Diabetic rats. At 6 h after STZ injection, a transient phase of portal hyperinsulinaemia and hypoglycaemia occurred, followed from 30 h onwards by the typical portal hyperglycaemia and insulinopenia of diabetic rats (Table 1).

To verify that STZ-injected rats were unable to secrete insulin, a glucose-induced insulin-secretion test was performed 30 h after STZ injection. A glucose solution (200 mg/g body wt.) was injected through the left jugular vein. Blood (300 μl) was sampled from the right jugular vein before and 5 min after glucose injection. Blood was immediately centrifuged, and plasma was frozen at −20 °C until plasma glucose and insulin

Table 2. Quantification by scanning densitometry of Northern blots of liver GLUT-2, GK and PEPCK mRNA in diabetic rats

Some diabetic rats were infused with either phlorizin (STZ + PHLO) from 18 to 72 h or insulin (STZ + INS) from 18 to 48 h and were compared with their respective diabetic rats (STZ 72 h and STZ 48 h). Some insulin-infused diabetic rats had their insulin infusion stopped after 48 h. They were studied 6 and 30 h after insulin withdrawal. Results are expressed as means ± S.E.M. of 4–6 rats in each group. The values in non-diabetic rats were taken as 100%. *Difference significantly different ($P < 0.05$) from control values. †Difference significantly different ($P < 0.05$) from STZ 72 h. ‡Difference significantly different ($P < 0.05$) from STZ 48 h. §Difference significantly different ($P < 0.05$) from STZ + INS.

		Time after STZ				Time after insulin withdrawal	
mRNA	Controls	48 h	72 h	STZ + PHLO 72 h	STZ + INS 48 h	6 h	30 h
GLUT-2	100 ± 15	205 ± 12*	213 ± 17*	111 ± 12†	75 ± 9‡	118 ± 21§	180 ± 26§
GK	100 ± 18	< 5*	< 5*	< 5	110 ± 15‡	< 5§	< 5§
PEPCK	100 ± 8	195 ± 15*	210 ± 22*	220 ± 25	73 ± 12‡	85 ± 18	203 ± 17§

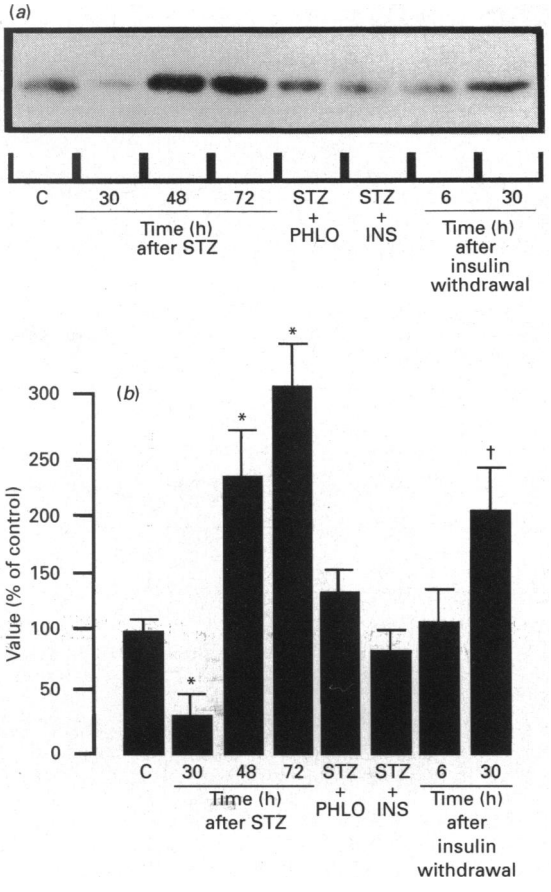


Fig. 3. (*a*) Representative autoradiograph of Western blots and (*b*) quantification by scanning densitometry of Western blots of GLUT-2 protein in liver membranes from control and diabetic rats

(*a*) Some diabetic rats were infused with either phlorizin (STZ+PHLO) from 18 to 72 h or insulin (STZ+INS) from 18 to 48 h and were compared with the corresponding diabetic rats (STZ 72 h and STZ 48 h). Some insulin-infused diabetic rats had their insulin infusion stopped after 48 h. They were studied 6 and 30 h after insulin withdrawal. In (*b*) results are expressed as means ± S.E.M. of 4–6 rats in each group. The values in non-diabetic rats were taken as 100%. *Difference significantly different ($P < 0.05$) from control values. †Difference significantly different ($P < 0.05$) from STZ+INS values. Abbreviation: C, control.

determinations. Plasma insulin concentrations in STZ-treated rats injected with glucose remained at the low basal value (< 5 μ-units/ml), despite very high plasma glucose concentration (> 20 mmol/l) (results not shown).

Phlorizin-treated diabetic rats. Some diabetic rats were infused with phlorizin 18 h after STZ injection. The rate of phlorizin infusion was adjusted to maintain the venous-blood glucose concentration at a mean value of 7.2 ± 0.4 mmol/l from 18 h to 72 h (Table 1). Portal plasma insulin concentration in phlorizin-treated diabetic rats (< 5 μ-units/ml) was not statistically different from the untreated diabetic rats (Table 1).

Insulin-treated diabetic rats. Some diabetic rats were infused with insulin 18 h after STZ injection. The rate of insulin infusion was adjusted to maintain the blood glucose concentration at a mean value of 6.6 ± 0.3 mmol/l from 18 h to 48 h (Table 1). The mean portal plasma insulin concentration reached during insulin infusion was 40 ± 8 μ-units/ml, i.e. a physiological concentration (Table 1). When insulin infusion was stopped, a rapid decrease in portal plasma insulin concentration and a marked hyperglycaemia occurred (Table 1). This allowed us to study short-term effects of insulin deprivation and hyperglycaemia on GLUT-2, GK and PEPCK mRNA concentrations.

Changes in GLUT-2, GK and PEPCK mRNA concentrations

GLUT-2, GK and PEPCK mRNA concentrations in liver of fed non-diabetic rats were used as controls. These values were considered as 100% and were used as initial value during the time-course experiments. GLUT-2 and PEPCK mRNA concentrations decreased by 90% as soon as 6 h after STZ injection, in parallel with the transient hyperinsulinaemia (Figs. 1*a* and 1*b*). They increased after 30 h to reach a level 2-fold higher than in non-diabetic rats after 48 h and later (Figs. 1*a* and 1*b*). In contrast, GK mRNA concentrations remained elevated 6 h after STZ injection and decreased rapidly after 18 h to reach very low level after 48 h (Figs. 1*a* and 1*b*).

Normalization of blood glucose concentration by phlorizin treatment restored liver GLUT-2 mRNA concentration to that found in non-diabetic rats (Fig. 2, Table 2), but did not modify the high level of liver PEPCK mRNA and the low level of GK mRNA found in diabetic rats (Fig. 2, Table 2).

In insulin-treated diabetic rats, liver GLUT-2 and PEPCK mRNA concentrations were 25% of those in untreated diabetic rats, and GK mRNA was markedly induced (Fig. 2, Table 2). At 6 h after insulin infusion was withdrawn, GLUT-2 and PEPCK mRNA concentrations rapidly increased and GK mRNA concentrations disappeared (Fig. 2, Table 2).

Changes in GLUT-2 protein concentration

GLUT-2 protein concentration was decreased by 70% after 30 h and then increased to a level 2–3-fold higher than the control values (Figs 3*a* and 3*b*). Phlorizin and insulin treatment of diabetic rats prevented the increase in GLUT-2 protein concentration (Figs. 3*a* and 3*b*). When insulin treatment was stopped, an increase in GLUT-2 protein concentration was observed after 30 h (Figs. 3*a* and 3*b*).

DISCUSSION

Glucose homoeostasis is markedly disturbed in the liver of diabetic rats. Our data confirm that the well-known hyperglycaemia, hyperglucagonaemia and hypoinsulinaemia that occur 30 h after STZ injection in diabetic rats is associated with a decrease in liver GK mRNA (Iynedjian *et al.*, 1988; Spence, 1983; Sibrowski & Seitz, 1984), an increase in liver PEPCK mRNA (Beale *et al.*, 1984) and a slight increase or an unchanged level of liver GLUT-2 mRNA (Thorens *et al.*, 1988; Oka *et al.*, 1990; Yamamoto *et al.*, 1991). Time-course experiments show that early portal hyperinsulinaemia and hypoglycaemia in STZ-treated rats are accompanied by a decrease in GLUT-2 mRNA and PEPCK and by a slight increase in GK mRNA. This is in agreement with the well-known effect of hyperinsulinaemia on GK and PEPCK expression (Cimbala *et al.*, 1982; Spence, 1983; Beale *et al.*, 1984; Iynedjian *et al.*, 1988). The fall in GLUT-2 mRNA in response to hyperinsulinaemia was unexpected from previous work in diabetic rats. Recent experiments from our laboratory have also shown that GLUT-2 mRNA concentrations are decreased by hyperinsulinaemia during a 6 h euglycaemic clamp performed in 24 h-fasted rats (Postic *et al.*, 1992). Moreover, the early fall in GLUT-2 mRNA is followed by a decrease in GLUT-2 protein concentration at 30 h. Thus data strongly suggest that euglycaemic hyperinsulinaemia inhibits liver GLUT-2 gene expression. About 30 h after STZ injection, PEPCK and GLUT-2 mRNA increase, and GK mRNA decreases, to reach after 48 h the levels reported previously in the liver of diabetic rats (Spence, 1983; Beale *et al.*, 1984; Sibrowski & Seitz, 1984; Iynedjian *et al.*, 1988). After 48 and 72 h

GLUT-2 protein levels are increased in liver of diabetic rats. To study the relative roles of hyperglycaemia and hypoinsulinaemia in the changes in GLUT-2 mRNA and protein levels, we have treated diabetic rats with phlorizin and insulin. The 2-fold increase in GLUT-2 mRNA and protein in liver of diabetic rats is corrected by normalization of blood glucose concentration by phlorizin, suggesting that hyperglycaemia stimulates GLUT-2 gene expression. This is in agreement with recent experiments showing that GLUT-2 mRNA is increased in the presence of high glucose concentrations in cultured rat hepatocytes (Asano *et al.*, 1992) and by experiments *in vivo* in which liver GLUT-2 mRNA does not decrease in response to hyperinsulinaemia in 24 h-fasted rats clamped at hyperglycaemic levels (Postic *et al.*, 1992). The inhibitory effect of insulin on GLUT-2 gene expression is further underlined by the fact that physiological insulin infusion decreases GLUT-2 mRNA and protein in the liver of diabetic rats, the level reached being 50 % lower than in phlorizin-treated diabetic rats. Moreover, when insulin infusion was stopped, GLUT-2 mRNA and protein concentrations rapidly returned to 'diabetic' levels.

The correction of hyperglycaemia by phlorizin in diabetic rats did not modify GK and PEPCK mRNA. Previous studies in cultured rat hepatocytes have shown that the expression of GK mRNA was not affected by glucose concentrations (Iynedjian *et al.*, 1989; Narkewicz *et al.*, 1990), but that PEPCK gene expression was inhibited by high glucose concentrations (Meyer *et al.*, 1991). However, in the latter study, very high glucose concentrations were used, and the physiological meaning of that experiment is not obvious. The regulatory effects of insulin on PEPCK and GK gene expression are further underlined by the fact that physiological insulin infusion decreases PEPCK mRNA and increases GK mRNA in the liver of diabetic rats. Moreover, when insulin infusion was stopped, PEPCK mRNA rapidly increased, and GK mRNA rapidly decreased, to diabetic levels.

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