Diabetologia (1993) 36: 292–298

Diabetologia
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Phlorizin treatment of diabetic rats partially reverses the abnormal expression of genes involved in hepatic glucose metabolism

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Summary. Liver insulin resistance and glucagon-stimulated hepatic glucose production are characteristics of the diabetic state. To determine the potential role of glucose toxicity in these abnormalities, we examined whether phlorizin treatment of streptozotocin-diabetic rats resulted in altered expression of genes involved in key steps of hepatic glucose metabolism. By inhibiting renal tubular glucose reabsorption, phlorizin infusion to diabetic rats induced normoglycaemia, did not significantly alter low circulating insulinaemia, but caused a marked decrease in hyperglucagonaemia. Glucokinase and L-type pyruvate kinase mRNA levels were reduced respectively by 90% and 70% in fed diabetic rats, in close correlation with changes in enzyme activities. Eighteen days of phlorizin infusion partially restored glucokinase mRNA and activity (40% of control levels), but had no effect on L-type pyruvate kinase mRNA and activity. In contrast to the glycolytic enzymes, mRNA and activity of the gluconeogenic enzyme, phospoenolpyruvate carboxykinase were increased (10- and 2.2-fold, respectively) in fed diabetic rats. Phlorizin administration decreased phospoenolpyruvate carboxykinase mRNA to values not different from those in control rats, while phospoenolpyruvate carboxykinase activity remained 50% higher than that in control rats. The 50% rise in liver glucose transporter (GLUT 2) mRNA and protein, produced by diabetes, was also corrected by phlorizin treatment. In conclusion, we propose that phlorizin treatment of diabetic rats may induce a partial shift of the predominating gluconeogenesis, associated with hepatic glucose overproduction, into glycolysis, by correction of impaired pre-translational regulatory mechanisms. This could be essentially mediated through improved pancreatic alpha-cell function and subsequent lowering of hyperglucagonaemia. These observations suggest that glucagon-stimulated hepatic glucose production may result, in part, from glucose toxicity.

Key words: Diabetes mellitus, phlorizin, glucagon, hepatic glucose metabolism, gene expression.

Defects in insulin secretion and insulin action are major features of the pathogenesis of both Type 1 (insulin-dependent) and Type 2 (non-insulin-dependent) diabetes mellitus in man, and similar disturbances have been shown in animal models of diabetes. It is well-established that an initial defect in insulin secretion can lead to the development of insulin resistance [1]. The latter appears first in the liver, then in peripheral tissues of rats made diabetic by streptozotocin injection [2]. During the last decade, it has been recognized that chronic hyperglycaemia itself may play a pathogenic role in diabetes by maintaining a self-perpetuating cycle of metabolic deterioration and the concept of glucose toxicity has emerged [3, 4]. The first evidence presented was that hyperglycaemia per se may attenuate the secretory response of pancreatic islet cells to glucose in some animal models of diabetes [5–7]. It has been further demonstrated that chronic hyperglycaemia may impair the effects of insulin on glucose uptake by peripheral tissues [3, 8–11] and on glucose production by the liver [10].

It has been suggested that, in insulin-deficient rats, hyperglycaemia-induced peripheral insulin resistance involved a down-regulation of the glucose transporter isoform GLUT 4 (decreased number in the plasma membrane) or an impairment of its intrinsic activity in muscle and adipose tissue or both [12, 13]. The mechanisms underlying hyperglycaemia-induced liver insulin resistance have not yet been determined.

Phlorizin treatment of diabetic rats, by inhibiting renal tubular glucose reabsorption, leads to near normal fed plasma glucose levels without significantly altering low fed plasma insulin levels. Such treatment has been shown to restore insulin action not only in peripheral tissues [9–11], but also in liver [10] of insulin-deficient animals. In the present study, we administered phlorizin as a continuous subcutaneous infusion to streptozotocin-diabetic rats

for 18 days. Then, we examined whether mere correction of hyperglycaemia by glucuresis reversed the abnormal expression of genes involved in hepatic glucose metabolism. This was achieved by measuring mRNA levels of glycolytic [glucokinase (GK) and L-type pyruvate kinase (L-PK)] and gluconeogenic [phosphoenolpyruvate carboxykinase (PEPCK)] enzymes, and of the glucose transporter isoform (GLUT 2) in liver of phlorizin-treated rats.

Materials and methods

Animals and experimental design

Male Wistar rats (8 weeks old; 224 ± 1 g) were purchased from IFFA Credo (L'Abresle, France). All rats received ad libitum a standard laboratory chow (Extralabo M-25; Ets. Pietrement, Provins, France; % of total energy (calories/calories): 60 % carbohydrate, 11 % fat, 29 % protein) and were housed in individual cages at a constant temperature (22 °C). A 12-h-light-dark cycle was set up (lights on 03.00–15.00 hours) with liver samples taken during the absorptive state of the animals (between 08.00–10.00 hours) at the end of the study.

The animals were divided into three experimental groups: non-diabetic control rats (C; $n = 5$); untreated diabetic rats (D; $n = 5$); diabetic rats treated with phlorizin (PHL; $n = 5$). Non-ketotic diabetes was induced by an i. v. injection of streptozotocin (45 mg/kg body weight) into a tail vein. Streptozotocin (Sigma, St Louis, Mo., USA) was dissolved in cold 0.1 mol/l citrate buffer (pH 4.5) immediately before use. Control animals received only the buffer. Five days after injection of streptozotocin, the diabetic rats were assigned to untreated or treated groups. The two groups of diabetic rats were matched for pre-treatment body weight (D: 249 ± 3 g, PHL: 240 ± 4 g, $p > 0.05$) and for pre-treatment fed plasma glucose levels (D: 25.6 ± 1.4 mmol/l, PHL: 26.2 ± 1.7 mmol/l, $p > 0.05$). The treated group received phlorizin (Sigma, 0.4 g/kg body weight per day, dissolved in propylene glycol) as a subcutaneous injection in two equally divided doses during the first 3 days, and thereafter as a continuous subcutaneous infusion via an implantable mini-pump. The osmotic mini-pump allowed for continuous inhibiton of renal tubular resorption of glucose and maintenance of a tighter glycaemic control. This pump (Alzet model 2 ML1; Alza Corp., Palo Alto, Calif., USA) was implanted under light ether anaesthesia, removed after 1 week of use and replaced by a second pump to obtain a 18-day phlorizin exposure period. Animals from the three groups were handled frequently to minimize non-specific reactions that could occur during blood sampling.

On several occasions, tail vein blood was collected from conscious fed animals for determination of plasma glucose and insulin levels. Plasma glucagon concentrations were also measured on days 5 and 17 of the study. Samples were collected into ice-cold heparinized tubes which were rapidly centrifuged. Plasma glucagon was protected from degradation by a trypsin inhibitor, Iniprol (Laboratoires Choay, Paris, France).

After 18 days of treatment, animals were killed between 08.00–10.00 hours (i. e. in the absorptive state). The liver was immediately removed, frozen in liquid nitrogen and stored at −80 °C for subsequent mRNA extraction, crude membrane preparation and enzyme activity measurement.

RNA extraction and Northern blot analysis

Total RNA was isolated by a single extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture [14]. The concentration of RNA was determined by absorbance at 260 nm. All samples had a 260/280 absorbance ratio of about 2. For Northern blot analysis, RNA (30 µg) was denaturated in a solution containing 2.2 mmol/l formaldehyde and 50 % formamide (volume/volume) by heating at 95 °C for 2 min. RNA was then size-fractionated by 1 % agarose gel electrophoresis, transferred to a Hybond-N membrane (Amersham Int., Amersham, Bucks, UK) and cross-linked by ultraviolet irradiation. The integrity and relative amounts of RNA were assessed by methylene blue staining of the blot.

The GK, L-PK, PEPCK and GLUT 2 cDNA probes were kindly supplied by Drs. P. Iynedjian [15], A. Kahn [16], R. W Hanson [17] and B. Thorens [18], respectively. Probes were labelled with ^{32}P using the Megaprime labelling system kit (Amersham). Hybridizations with L-PK, PEPCK or GLUT 2 probes were carried out in medium containing denatured salmon sperm DNA (0.3 mg/ml), 42 % deionized formamide, 7.5 % dextran sulphate, 8 × Denhardt's solution (Denhardt 1 ×:0.2 g/l polyvinylpyrrolidone, 0.2 g/l bovine serum albumin, 0.2 g/l ficoll 400), 40 mmol/l-Tris/HCl (pH 7.5), 0.1 % sodium pyrophosphate and 1 % sodium dodecyl sulphate (SDS) at 42 °C overnight. Hybridization with GK probe was performed in 3 × sodium saline phosphate ethylenediamine tetraacetic acid (SSPE) buffer, 1 % SDS, 1 % glycine, 0.2 % ficoll, 0.2 % polyvinylpyrrolidone, 10 % polyethylene glycol and denaturated salmon sperm DNA (0.2 mg/ml) at 65 °C overnight. The membrane was washed two times for 30 min in 2 × sodium saline citrate (SSC) buffer/0.1 % SDS at 42 °C and once for 15–30 min in 0.1–0.5 × SSC/0.1 % SDS at 55 °C (PEPCK, GLUT 2) or 65 °C (L-PK). The membrane hybridized with GK was washed two times for 20 min in 2 × SSPE/1 % SDS and once for 15 min in 0.5 × SSPE/1 % SDS at 65 °C. The filters were then exposed to Fuji X-Ray films for 7–48 h at −80 °C with intensifying screens.

Intensity of the mRNA bands on the blots was quantified by scanning densitometry (Hoefer, San Francisco, Calif., USA).

Membrane preparation and Western blot analysis

Total liver membranes were prepared according to Thorens et al. [18]. Briefly, tissue (± 200 mg) was homogenized with 10 strokes of a motor-driven Teflon pestle in 20 volumes of 0.25 mol/l sucrose/25 mmol/l Hepes buffer (pH 7.4) containing 3 mmol/l dithiothreitol, 0.26 U/ml aprotinin, 0.1 mmol/l phenylmethylsulphonyl fluoride. This homogenate was centrifuged for 10 min at 8000 *g* and the supernatant was centrifuged again for 20 min at the same speed. The cytosol was then centrifuged for 40 min at 150000 *g* and the membrane pellet was resuspended in phosphate-buffered saline (8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l NaH_2PO_4, 0.24 g/l KH_2PO_4; pH 7.4). Protein content was measured by the method of Bradford [19] (Bio-Rad, Munich, FRG), using bovine serum albumin as standard.

Equal amounts of membrane proteins (100 µg) were solubilized in Laemmli buffer [20], submitted to a 12 % SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, FRG). Rainbow protein markers were used as molecular weight standards and also to assess the efficiency of the transfer. Filters were then processed as previously described [18]. After blocking, the membranes were incubated with a 10^{-2} dilution of anti-peptide antibody directed against GLUT 2 (a gift from Dr B. Thorens). The detection was performed with 0.1 µCi/ml ^{125}I-protein A, followed by autoradiography and subsequent quantification by scanning densitometry.

Measurement of enzyme activities

All enzyme activities were carried out at 37 °C. GK (EC 2.7.1.1) activity was determined using a spectrophotometric assay [21] by subtracting the hexokinase activity measured at 0.5 mmol/l glucose from the activity measured at 100 mmol/l glucose. Results were expressed as µmol NADH produced · min^{-1} · g $liver^{-1}$. L-PK (EC 2.7.1.40) was measured according to Blair et al. [22] at a subsaturating concentration of phosphoenolpyruvate (PEP) (1.3 mmol/l) (active form of the enzyme) and at saturating concentration of PEP (6.6 mmol/l) (maximal activity of the enzyme). Results were expressed as µmol NADH

Table 1. Body weight gain and plasma glucose, insulin and glucagon levels in fed rats of the three experimental groups

	During treatment			End of treatment
	Body weight gain (g/day)	Plasma glucose (mmol/l)	Plasma insulin (ng/ml)	Plasma glucagon (pg/ml)
Control	4.2 ± 0.3	7.2 ± 0.2	3.67 ± 0.29	191 ± 8
Diabetic	2.3 ± 0.5[a]	28.8 ± 1.2[b]	1.39 ± 0.18[b]	338 ± 24[b]
Diabetic + phlorizin	2.7 ± 0.6[a]	9.0 ± 0.6[c]	1.78 ± 0.14[b]	191 ± 17[c]

[a] $p < 0.05$, [b] $p < 0.01$ vs control rats; [c] $p < 0.01$ vs diabetic rats
Values are means ± SEM for five rats in each group. Diabetes was induced by an i. v. injection of streptozotocin 5 days before the treatment. A group of diabetic rats was untreated; another group received subcutaneous infusion of phlorizin from day 0 to day 18. Control rats were not injected with streptozotocin (only with citrate buffer) and did not receive phlorizin. Three samples were taken for plasma glucose and insulin measurements during the treatment period (days 5, 12, 17); the values obtained were averaged for each rat. Values of plasma glucagon are those determined at the end of the study (day 17)

Table 2. Effects of phlorizin treatment on L-type pyruvate kinase (L-PK), glucokinase (GK), phosphoenolpyruvate carboxykinase (PEPCK) activities and on GLUT 2 protein in liver of diabetic rats

	L-PK		GK	PEPCK	GLUT 2 Protein
	PEP 1.3 mmol/l	PEP 6.6 mmol/l			
	μmol of substrate transformed per min per g of liver				% of controls
Control	40.5 ± 6.3	65.6 ± 5.7	3.64 ± 0.30	1.11 ± 0.09	100 ± 21
Diabetic	8.9 ± 1.5[b]	17.8 ± 2.4[b]	0.26 ± 0.02[b]	2.45 ± 0.19[b]	165 ± 37
Diabetic + phlorizin	12.4 ± 1.1[b]	24.0 ± 1.7[b]	1.58 ± 0.20[b,c]	1.68 ± 0.21[a,c]	115 ± 21

[a] $p < 0.05$, [b] $p < 0.01$ vs control rats; [c] $p < 0.01$ vs diabetic rats
Values are means ± SEM for five rats in each group. The animals were killed in the fed state after 18 days of treatment. GLUT 2 protein was measured by western blot analysis, quantified by scanning densitometry and expressed as a percentage of control rats. PEP, Phosphoenolpyruvate

oxidized · min^{-1} · g $liver^{-1}$. PEPCK (EC 4.1.1.32) activity was determined in liver cytosolic fractions using the $NaH[^{14}C]O_3$ fixation assay of Chang and Lane [23]. Results were expressed as μmol $NaH[^{14}C]O_3$ fixed · min^{-1} · g $liver^{-1}$.

Analytical procedures

Plasma glucose was measured by a glucose oxidase method (Peridochrom; Boehringer-Mannheim, Mannheim, FRG). Plasma insulin was determined by a double-antibody radioimmunoassay, using rat insulin as standard (Novo Research Institute, Bagsvaerd, Denmark). Plasma glucagon was measured by a radioimmunoassay using a charcoal separation step and the antiserum 04A, which is considered to be specific for pancreatic glucagon (Dr. R. H. Unger, Dallas, Tx., USA).

Statistical analysis

Results are given as the mean ± SEM for the indicated number of rats. Comparisons between control, diabetic, and phlorizin-treated-diabetic rats were carried out by analysis of variance followed by the Newman-Keuls test for multiple comparisons [24]. Student's t-test for paired data was used to evaluate the time course of phlorizin effect on plasma insulin levels. Differences were considered statistically significant at $p < 0.05$.

Results

Diabetic rats gained weight at a much lower rate than controls. This growth retardation was not improved by phlorizin treatment (Table 1).

Average plasma glucose levels in fed diabetic rats were above 25 mmol/l and their plasma insulin levels were decreased by more than 50%. Phlorizin administration to diabetic rats resulted in a normalization of plasma glucose concentrations (Table 1). The correction of hyperglycaemia was observed as early as after 5 days of treatment and persisted until the end of the experiment. This effect was not due to a rise in plasma insulin levels, which did not change throughout the study (day 5 vs day 17, $p > 0.05$ by paired t-test) and remained low in fed phlorizin-treated diabetic rats (Table 1). Plasma glucagon levels were increased by 75% in diabetic animals. This rise was totally prevented by 17 days of phlorizin administration (Table 1). The glucagon-lowering effect induced by phlorizin was not observed after 5 days of treatment (data not shown).

Protracted hyperglycaemia in diabetic rats was associated with overt polydipsia: their daily fluid consumption was four-fold greater than in control rats (D: 188 ± 12 ml/day vs C: 40 ± 4 ml/day, $p < 0.01$). Although reduced, fluid intake in phlorizin-treated diabetic rats (107 ± 2 ml/day) remained two-fold higher than in controls ($p < 0.01$), which probably reflects the increased urinary losses of glucose produced by the treatment.

To determine whether relief of hyperglycaemia by phlorizin glucuresis was associated with altered expression of genes involved in key steps of hepatic glucose metabolism, we measured mRNA levels of glycolytic and gluconeogenic enzymes, and of the GLUT 2 transporter isoform. Yields of total RNA in liver did not differ between the three groups (C: 4.2 ± 0.4 mg/g; D: 5.1 ± 0.6 mg/g, PHL: 4.7 ± 0.3 mg/g). The abundance of mRNAs encoding the glycolytic enzymes, L-PK and GK,

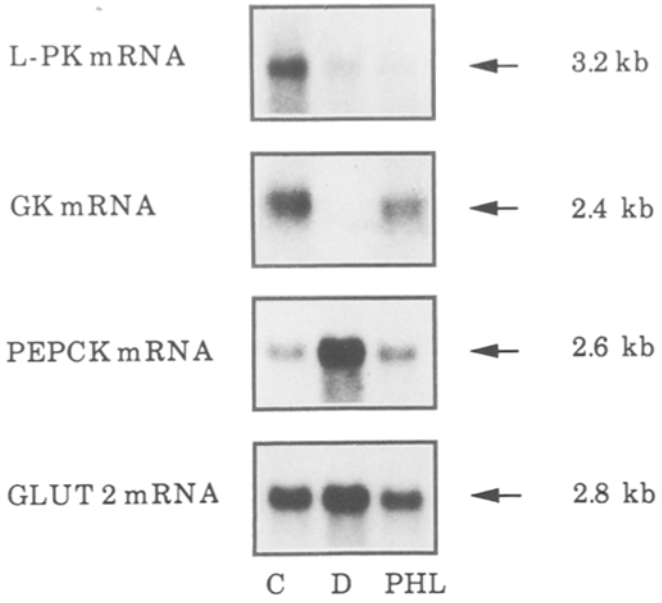

Fig. 1. Northern-blot analysis of glucokinase (GK), L-type pyruvate kinase (L-PK), phosphoenolpyruvate carboxykinase (PEPCK) and GLUT 2 transporter mRNA in liver from control (C), untreated diabetic (D) and phlorizin-treated diabetic (PHL) rats. All lanes were loaded with 30 μg of total RNA and the filter was hybridized successively with the different radiolabelled cDNA probes. This figure is representative of five different rats in each group. kb, kilobases

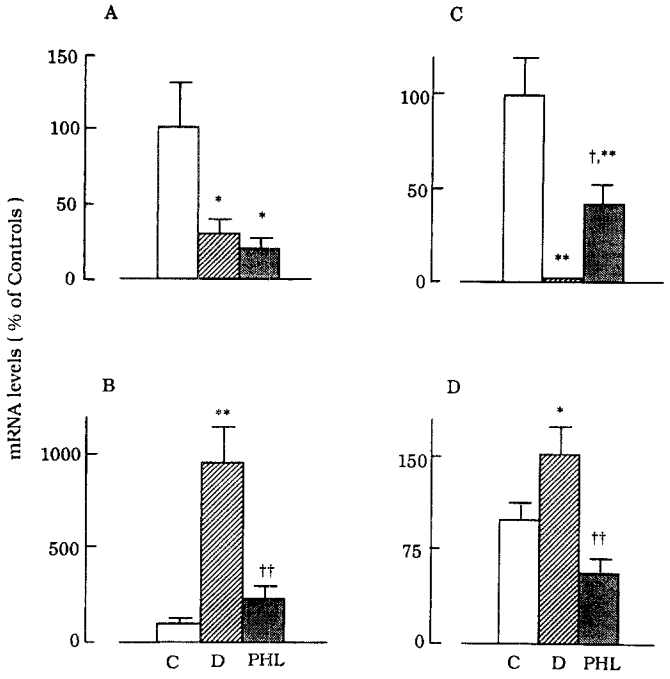

Fig. 2 A–D. Effects of phlorizin treatment on **A**, L-type pyruvate kinase, **B**, phosphoenolpyruvate carboxykinase, **C**, glucokinase, and **D**, GLUT 2 transporter mRNA levels in liver of diabetic rats. Values are means ± SEM for five control (C), untreated diabetic (D) and phlorizin-treated diabetic (PHL) rats. mRNA levels were quantified by scanning densitometry of autoradiographic signals obtained from a Northern blot hybridized successively with the different radiolabelled cDNA probes. Results were expressed as percentages of values in control rats. Statistical significance of differences (analysis of variance followed by Newman-Keuls test): $^{*}p < 0.05$, $^{**}p < 0.01$ vs control rats; $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ vs untreated diabetic rats

was assessed by Northern blot analysis. L-PK and GK mRNA transcripts (3.2 and 2.4 kilobases (kb), respectively) were barely detectable in diabetic liver (Fig. 1). Quantification of autoradiographic signals showed that L-PK and GK mRNAs were decreased, respectively by 70% and 90%, in diabetic rats compared with control rats (Fig. 2). The activities of L-PK (at submaximal and maximal PEP concentrations) and GK were lowered accordingly (Table 2). Phlorizin treatment given to streptozotocin-diabetic rats for 18 days did not modify L-PK mRNA and activity. However, this treatment restored GK mRNA and activity to 41% and 43% of control levels, respectively. Compared to untreated diabetic rats, GK mRNA and activity were increased 20- and five-fold, respectively, in liver of phlorizin-treated rats (Figs. 1 and 2, Table 2).

Diabetes induced a 10-fold rise in mRNA levels of the gluconeogenic enzyme, PEPCK (Figs. 1 and 2), and a 2.2-fold rise in the enzyme activity, as compared to control rats (Table 2). Phlorizin administration decreased PEPCK mRNA to values not significantly different from those in control rats, while PEPCK activity remained only slightly higher than that in controls (50%) (Figs. 1 and 2, Table 2).

Glucose transport in liver is mostly mediated by GLUT 2, the glucose transporter isoform which is involved in either net glucose uptake or release depending on the prevailing metabolic conditions. Examination of GLUT 2 mRNA transcript (2.8 kb) abundance on Northern blots indicated that levels of these liver transcripts were increased by 50% in diabetic liver (Figs. 1 and 2). The amount of GLUT 2 protein was measured by Western blot analysis in crude liver membranes. GLUT 2 protein levels were slightly increased by diabetes (Table 2). Altered abundance of GLUT 2 mRNA and protein was corrected upon treatment of diabetic rats with phlorizin.

Discussion

We confirmed that chronic administration of phlorizin to streptozotocin-diabetic rats normalized their fed plasma glucose levels without significantly altering their fed plasma insulin levels. We further showed that this treatment also corrected their fed hyperglucagonaemia and partially corrected the abnormal expression of genes involved in hepatic glucose metabolism.

In a previous study, no change in basal plasma glucagon levels was observed in diabetic rats after chronic phlorizin treatment [25]. However, the partially pancreatectomized rat model was used which did not exhibit absolute hyperglucagonaemia [25] due to a 70% decrease in its pancreatic glucagon content [5]. In the present work, hyperglucagonaemia is clearly present in streptozotocin-diabetic rats. Several mechanisms could theorically contribute to the glucagon-lowering effect induced by phlorizin in these animals. First, a direct action of phlorizin on alpha cells. This is unlikely, since direct perfusion of the drug into the isolated pancreas did not alter glucagon secretion [6]. Second, the circulatory arrangement within islets exposes alpha cells to the highest circulating insulin concentrations in the body and facilitates a role for insulin as an in-

hibiting factor for glucagon release [26]. Fed plasma insulin concentrations, measured throughout this study, did not significantly increase in diabetic rats treated with phlorizin. This is in agreement with previous reports which further showed that phlorizin did not alter insulin secretion in response to a meal or an i.v. glucose bolus in similarly mildly insulin-deficient rats [7, 9, 10, 25]. However, minute restoration of glucose induced-insulin release cannot be excluded. Some restoration of beta-cell function, barely detectable by the tests mentioned above, has been observed during hyperglycaemic clamp studies performed in partially pancreatectomized rats chronically treated with phlorizin [7]. It should be emphasized, however, that in these studies, the plasma insulin response remained small, when considered in absolute terms, and did not exceed 15% of that in control rats [7]. Third, it has been suggested that alpha cells have a glucose-sensing system that functions independently of insulin and beta cells and that this system may be attenuated by chronic hyperglycaemia. A rise in glucose concentration does not suppress glucagon release in the insulin-deficient diabetic state [27], which it normally does. Strong evidence that diabetic alpha-cell "blindness" to glucose can be reversed, despite continuing insulin deficiency, by mere correction of hyperglycaemia with phlorizin, has been obtained in totally insulin-deprived dogs [6]. The normal decrease in plasma glucagon levels in response to a physiological rise in glycaemia was restored in phlorizin-treated (normoglycaemic) diabetic dogs [6]. Such a mechanism is likely to play a determining role in the normalization of glucagonaemia, as shown in the present study in long-term phlorizin-treated diabetic rats.

It has been reported that chronic administration of glucagon to normal rats modified a number of hepatic enzyme activities (including L-PK and PEPCK), mainly by affecting enzyme levels [28]. Since chronic hyperglucagonaemia opposes the suppressive effect of insulin on hepatic glucose production [29] and since liver insulin resistance in diabetic rats has been shown to be corrected by phlorizin treatment [10], we examined whether such a treatment could reverse the abnormal expression of several genes involved in key enzymes of hepatic glucose metabolism. Phlorizin administration to diabetic rats partially restored GK mRNA levels (40% of control values; 20-fold rise compared with untreated diabetic rats), reversed PEPCK mRNA levels, but had no effect on L-PK mRNA levels. All these effects of phlorizin may be related to hormonal changes (mainly lowering of hyperglucagonaemia) or correction of hyperglycaemia or both. Evidence has been presented that the mRNA levels of glycolytic enzymes (GK and L-PK) are increased by insulin and decreased by glucagon both in vivo [30–32] and in vitro [32–34]. In cultured hepatocytes, the inhibitory effect of glucagon dominates over the stimulatory effect of insulin in the control of the expression of these enzymes [33, 34]. Thus, correction of hyperglucagonaemia may allow, in the presence of low – but still detectable – plasma insulin levels, a partial re-induction of GK mRNA in the liver of phlorizin-treated diabetic rats. In contrast to GK, whose transcription is glucose-independent [33], L-PK expression requires the presence of high glucose levels for its induction by insulin [34]. The lack of L-PK re-induction in phlorizin-treated diabetic rats could, therefore, be explained by the lowering of glycaemia, which prevented the effect due to the correction of hyperglucagonaemia.

Liver PEPCK gene expression is increased by glucagon and decreased by insulin both in vivo [35] and in vitro [32], and this regulation appears to be mainly glucose-independent [32]. It was first shown in hepatoma cells that the inhibitory effect of insulin on PEPCK transcription was predominant over the stimulatory effect of cAMP (second messenger of glucagon) [36]. However, hepatoma cells do not possess the enzyme pattern and hormone responsiveness of normal liver cells. In a more physiological model, namely primary cultures of hepatocytes, it was recently demonstrated that glucagon was predominant over insulin in the regulation of PEPCK expression [37]. Thus, the lowering of hyperglucagonaemia, again, may be the major mechanism which could account for the reduction of PEPCK expression in phlorizin-treated mildly insulin-deficient rats. One cannot preclude, however, that the reversal of GK and PEPCK mRNAs would have been less pronounced if more insulinopoenic rats were used.

Alterations in enzyme activities of treated diabetic rats paralleled changes in mRNA concentrations. In the fed state, PEPCK activity was however only partially restored, whereas PEPCK mRNA levels were decreased to values not significantly different from those in control rats. This may be due to the difference in half-lives, since it has been shown that, during refeeding, PEPCK activity decays more slowly ($t_{1/2} \approx 6$ h) [38] than the corresponding mRNA ($t_{1/2} \approx 1$ h) [35].

Although glycolytic and gluconeogenic fluxes were not measured in the present study, changes in enzyme activities are likely to reflect changes in their actual catalytic rates. Indeed, GK and PEPCK activities are not regulated by phosphorylation/dephosphorylation or by allosteric mechanisms. L-PK activity is markedly decreased by glucagon-mediated phosphorylation [22]. The active form of L-PK, which takes into account the phosphorylation state of the enzyme [22], has been measured in the current study. The changes in active form of L-PK paralleled the changes in maximal activity. On this basis, the present data strongly suggest that phlorizin treatment may partially shift liver gluconeogenesis into glycolysis in diabetic rats.

Glucose transport is not considered to be the rate-limiting step of hepatic glucose metabolism, and the major transporter GLUT 2 is involved in either net glucose uptake or release, depending upon the prevailing metabolic conditions [39]. In agreement with some [40, 41], but not all [42], previous reports, a 50% rise in GLUT 2 mRNA was observed in the liver of streptozotocin-diabetic rats. GLUT 2 protein was also slightly increased. This could contribute to the enhanced hepatic glucose output from diabetic liver [40]. Altered abundance of GLUT 2 mRNA and protein was corrected by phlorizin treatment of diabetic rats. In vivo experiments with primary cultured hepatocytes have shown that GLUT 2 gene was increased by high glucose levels [43, 44] and decreased by insulin [44]. Evidence that such a regulation also occurred in vivo has recently been provided [44, 45]. The potential regulatory role of glucagon on GLUT 2 has not yet been in-

vestigated. However, correction of hyperglycaemia in the presence of low circulating insulinaemia may contribute to understanding the effects produced by phlorizin treatment on GLUT 2 expression in diabetic rats.

In conclusion, elimination of chronic hyperglycaemia by phlorizin glucuresis may allow some metabolic compensation in diabetic liver. Long-term phlorizin treatment of diabetic rats may induce a partial shift of the predominating gluconeogenesis, associated with hepatic glucose overproduction, into glycolysis, by correction of impaired pre-translational regulatory mechanisms. This could be essentially mediated through improvement of pancreatic alpha-cell function and subsequent lowering of hyperglucagonaemia. These observations suggest that glucagon-stimulated hepatic glucose production results, in part, from glucose toxicity and reinforce the need for tight glycaemic control in diabetes.

Acknowledgements. We thank Drs. P. Iynedjian (Geneva, Switzerland), A. Kahn (Paris, France), R. W. Hanson (Cleveland, Ohio, USA) and B. Thorens (Lausanne, Switzerland) for kindly providing us with the cDNA probes and the antibody used in this study. We are grateful to Prof. R. Assan (Paris) for helpful suggestions and to Mr. M. Gerard for hormone assays. This work was supported in part by Fondation de la Recherche Médicale (Paris) and by a Grant 1.5.078.92 from the FNRS (Brussels). S. M. B. acknowledges receipt of a fellowship from the Institut National de la Santé et de la Recherche Medicale.

References

1. Reaven GM, Sageman WS, Swenson RS (1977) Development of insulin resistance in normal dogs following alloxan-induced insulin deficiency. Diabetologia 13: 459–462
2. Blondel O, Portha B (1989) Early appearance of in vivo insulin resistance in adult streptozotocin-injected rats. Diabète Metab 15: 382–387
3. Unger RH, Grundy S (1985) Hyperglycaemia as an inducer as well as a consequence of impaired islet cell function and insulin resistance: implications for the management of diabetes. Diabetologia 28: 119–121
4. Yki-Järvinen H (1990) Acute and chronic effects of hyperglycaemia on glucose metabolism. Diabetologia 33: 579–585
5. Bonner-Weir S, Trent DF, Weir GC (1983) Partial pancreatectomy in the rat and subsequent defect in glucose-induced insulin release. J Clin Invest 71: 1544–1553
6. Starke A, Grundy S, McGarry JD, Unger RH (1985) Correction of hyperglycemia with phlorizin restores the glucagon response to glucose in insulin-deficient dogs: implications for human diabetes. Proc Natl Acad Sci USA 82: 1544–1546
7. Rossetti L, Shulman GI, Zawalich W, De Fronzo RA (1987) Effect of chronic hyperglycemia on in vivo insulin secretion in partially pancreatectomized rats. J Clin Invest 80: 1037–1044
8. Yki-Järvinen H, Helve E, Koivisto VA (1987) Hyperglycemia decreases glucose uptake in type I diabetes. Diabetes 36: 892–896
9. Rossetti L, Smith D, Shulman GI, Papachristou D, De Fronzo RA (1987) Correction of hyperglycemia with phlorizin normalizes tissue sensitivity to insulin in diabetic rats. J Clin Invest 79: 1510–1515
10. Blondel O, Bailbe D, Portha B (1990) Insulin resistance in rats with non-insulin-dependent diabetes induced by neonatal (5 days) streptozotocin: evidence for reversal following phlorizin treatment. Metabolism 39: 787–793
11. Lisato L, Cusin I, Tiengo A, Del Prato S, Jeanrenaud B (1992) The contribution of hyperglycaemia and hypoinsulinaemia to the insulin resistance of streptozotocin diabetic rats. Diabetologia 35: 310–315
12. Kahn BB, Shulman GI, De Fronzo RA, Cushman SW, Rossetti L (1991) Normalization of blood glucose in diabetic rats with phlorizin treatment reverses insulin-resistant glucose transport in adipose cells without restoring glucose transporter gene expression. J Clin Invest 87: 561–570
13. Dimitrakoudis D, Ramlal T, Rastogi S, Vranic M, Klip A (1992) Glycaemia regulates the glucose transporter number in the plasma membrane of rat skeletal muscle. Biochem J 284: 341–348
14. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium-thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159
15. Iynedjian PB, Ucla C, Mach B (1987) Molecular cloning of glucokinase cDNA. Developmental and dietary regulation of glucokinase mRNA in rat liver. J Biol Chem 262: 6032–6038
16. Simon MP, Besmond C, Cottreau D et al. (1983) Molecular cloning of cDNA for rat L-type pyruvate kinase and aldolase B. J Biol Chem 258: 14576–14584
17. Yoo-Warren H, Monahan JE, Short J et al. (1983) Isolation and characterization of the gene coding for cytosolic phosphoenolpyruvate carboxykinase (GTP) from the rat. Proc Natl Acad Sci USA 80: 3656–3660
18. Thorens B, Sarkar HK, Kaback HR, Lodish HF (1988) Cloning and functional expression in bacteria of a novel glucose transporter present in liver, intestine, kidney, and B-pancreatic islet cells. Cell 55: 281–290
19. Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
20. Laemmli UK (1970) Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond) 227: 680–685
21. Newgard CB, Hirsch LJ, Foster DW, Mc Garry JD (1983) Studies on the mechanism by which exogenous glucose is converted into liver glycogen in the rat. J Biol Chem 258: 8046–8052
22. Blair JB, Cimbala MA, Foster JL et al. (1976) Hepatic pyruvate kinase. Regulation by glucagon, cyclic adenosine 3′:5′-monophosphate, and insulin in the perfused rat liver. J Biol Chem 251: 3756–3762
23. Chang HC, Lane MD (1966) The enzymatic carboxylation of phosphoenolpyruvate. II. Purification and properties of liver mitochondrial phosphoenolpyruvate carboxykinase. J Biol Chem 241: 2413–2420
24. Sokal RR, Rohlf FJ (1969) Biometry. The principles and practice of statistics in biological research. Freeman, San Francisco, pp 1–776
25. Rossetti L, Laughin MR (1989) Correction of hyperglycemia with vanadate, but not with phlorizin, normalizes in vivo glycogen repletion and in vitro glycogen synthase activity in diabetic skeletal muscle. J Clin Invest 84: 892–899
26. Unger RH (1983) Insulin-glucagon relationships in the defense against hypoglycemia. Diabetes 32: 575–583
27. Muller WA, Faloona GR, Aguilar-Parada E, Unger RH (1971) Abnormal alpha-cell function in diabetes. Response to carbohydrate and protein ingestion. N Engl J Med 283: 109–115
28. Gruppuso PA, Susa JB, Domenech M et al. (1983) Chronic hyperglucagonemia in rats: effects on insulin, substrates and hepatic enzymes of intermediary metabolism. Metabolism 32: 911–918
29. Del Prato S, Castellino P, Simonson DC, De Fronzo RA (1987) Hyperglucagonemia and insulin-mediated glucose metabolism. J Clin Invest 79: 547–556
30. Sibrowski W, Seitz HJ (1984) Rapid action of insulin and cyclic AMP in the regulation of functional mRNA coding for glucokinase in rat liver. J Biol Chem 259: 343–346
31. Vaulont S, Munnich A, Decaux JF et al. (1986) Transcriptional and post-transcriptional regulation of L-type pyruvate kinase gene expression in rat liver. J Biol Chem 261: 7621–7625
32. Granner D, Pilkis S (1990) The genes of hepatic glucose metabolism. J Biol Chem 265: 10173–10176

33. Iynedjian PB, Jotterand D, Nouspikel T, Asfari M, Pilot PR (1989) Transcriptional induction of glucokinase gene by insulin in cultured liver cells and its repression by the glucagon-cAMP system. J Biol Chem 264: 21824–21829
34. Decaux JF, Antoine B, Kahn A (1989) Regulation of the expression of the L-type pyruvate kinase gene in adult rat hepatocytes in primary culture. J Biol Chem 264: 11584–11590
35. Cimbala MA, Lamers WH, Nelson K, Monahan JE, Yoo-Warren H, Hanson RW (1982) Rapid changes in the concentration of phosphoenolpyruvate carboxykinase mRNA in rat liver and kidney. Effects of insulin and cyclic AMP. J Biol Chem 257: 7629–7636
36. Sasaki K, Cripe TP, Koch SR et al. (1984) Multihormonal regulation of phosphoenolpyruvate carboxykinase gene transcription. The dominant role of insulin. J Biol Chem 259: 15242–15251
37. Christ B, Nath A, Bastian H, Jungermann K (1988) Regulation of the expression of the phosphoenolpyruvate carboxykinase gene in cultured rat hepatocytes by glucagon and insulin. Eur J Biochem 178: 373–379
38. Hopgood MF, Ballard FJ, Reshef L, Hanson RW (1973) Synthesis and degradation of phosphoenolpyruvate carboxylase in rat liver and adipose tissue. Changes during a starvation-re-feeding cycle. Biochem J 134: 445–453
39. Mueckler M (1990) Family of glucose transporter genes. Implications for glucose homeostasis and diabetes. Diabetes 39: 6–11
40. Oka Y, Asano T, Shibasaki Y et al. (1990) Increased liver glucose-transporter protein and mRNA in streptozotocin-induced diabetic rats. Diabetes 39: 441–446
41. Yamamoto T, Fukumoto H, Koh G et al. (1991) Liver and muscle-fat type glucose transporter gene expression in obese and diabetic rats. Biochem Biophys Res Commun 175: 995–1002
42. Thorens B, Flier JS, Lodish HF, Kahn BB (1990) Differential regulation fo two glucose transporters in rat liver by fasting and refeeding and by diabetes and insulin treatment. Diabetes 39: 712–719
43. Asano T, Katagiri H, Tsukuda K et al. (1992) Upregulation of GLUT 2 mRNA by glucose, mannose, and fructose in isolated rat hepatocytes. Diabetes 41: 22–25
44. Leturque A, Postic C, Burcelin R, Pegorier JP, Loizeau M, Girard J (1992) Regulation by insulin and glucose of GLUT 2 mRNA in rat liver: in vivo and in vitro studies. Diabetes 41 [Suppl 1]: 44A
45. Burcelin R, Eddouks M, Kandé J, Assan R, Girard J (1992) Evidence that Glut-2 mRNA and protein concentrations are decreased by hyperinsulinaemia and increased by hyperglycaemia in the liver of diabetic rats. Biochem J 288: 675–679

Received: 7 September 1992
and in revised form: 23 November 1992

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