Insulin mediates the stimulation of pyruvate kinase by a dual mechanism

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A radioimmunoassay specific for liver pyruvate kinase was used to determine the mechanism(s) involved in the insulin stimulation of this enzyme activity in chronically diabetic rats. Rats, made diabetic with alloxan, were fed on a high-carbohydrate (50%-sucrose) fat-free diet and treated with insulin for 12, 36 or 60 h. Livers were removed at the various times, a piece was kept for determination of glycogen, and the remainder was homogenized. The 100 000 g supernatant was prepared and used for determination of pyruvate kinase activity and quantity. Glycogen increased to a maximum of approx. 7% by 12 h after insulin treatment, and was maintained at this elevated value for 60 h. Liver pyruvate kinase activity, which is depressed in diabetes, did not respond to insulin until 36 h of treatment, with a more substantial increase occurring by 60 h. Radioimmunoassay data indicated that the increase in activity was concomitant with a substantial increase in the quantity of the enzyme and a moderate increase in its specific activity. These results demonstrate that a dual mechanism, i.e. an increase in both the quantity and specific activity of the enzyme, regulates the insulin-mediated stimulation of liver pyruvate kinase in the diabetic rat.

The insulin-mediated control of liver pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) has been studied in a variety of systems. In normal rats, the pyruvate kinase activity is rapidly suppressed by gluconeogenic activators (Taunton *et al.*, 1974; Chan & Exton, 1978), which stimulate a cyclic AMP-dependent phosphorylation of the enzyme molecule (Ljungström *et al.*, 1974), and insulin re-activates by mediating a rapid dephosphorylation of the enzyme (Claus *et al.*, 1979). Additional studies in animals with experimentally induced diabetes have demonstrated that liver pyruvate kinase activity is diminished in diabetes and restored by insulin treatment (Weber *et al.*, 1965; Tanaka *et al.*, 1967; Chang & Schneider, 1971; Johnson & Veneziale, 1980). However, these investigators differ in their description of the kinetics of the insulin-mediated induction. Tanaka *et al.* (1967) reported that this enzyme activity in the diabetic animal increases with 6 h of insulin treatment, whereas Weber *et al.* (1965) demonstrate an increase in 24 h and Chang & Schneider (1971) in less than 48 h.

Furthermore, these reports differ with respect to the proposed mechanism of induction. Weber *et al.*

(1965) suggested that insulin stimulates the synthesis of liver pyruvate kinase, since actinomycin D or ethionine prevents any increase in enzyme activity, whereas Johnson & Veneziale (1980) detected no increase in the quantity of the enzyme after 96 h of insulin treatment of diabetic rabbits, though its activity is increased. They concluded that insulin does not induce the synthesis of liver pyruvate kinase, but rather that the hormone mediates a modification of existing enzyme molecules, resulting in an increase in the specific activity.

We now report in the present paper that in the chronically diabetic animal insulin mediates a delayed rise in liver pyruvate kinase activity that is due to a substantial increase in the quantity of the enzyme and a moderate increase in its specific activity. Additionally, we discuss the diabetic model and the specific requirements that these animals must satisfy.

Experimental

Animal treatment and tissue preparation

Diabetes was produced in male Sprague–Dawley rats with initial body weights of 170–195 g by intravenous injection of 4% (w/v) alloxan monohydrate (Eastman Organic Chemicals) at a dosage of 55 mg/

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kg body wt. Rats with plasma glucose concentrations in excess of 300mg/dl, determined by using hexokinase and glucose 6-phosphate dehydrogenase (Bergmeyer *et al.*, 1974), and with little or no weight gain were considered diabetic (Lazarow & Palay, 1946). These rats were maintained for 10 days after administration of alloxan to allow stabilization of the diabetic condition.

After 10 days, the diabetic rats were divided into two groups. One diabetic group was fed on a high-carbohydrate [50%-(w/w)-sucrose] fat-free diet (AIN-76; Zeigler Brothers, Gardners, PA, U.S.A.) for 60h. This served as a control group that provided information on the effect of the special diet on liver pyruvate kinase activity. The second diabetic group was fed on the same diet and given subcutaneous injections of 4 units each of regular insulin (Regular Iletin) and NPH insulin (NPH Iletin) every 12h for 12, 36 or 60h. All rats were killed at 09:00h at the specified times after the initiation of either diet or diet and insulin treatment. The livers were removed, weighed and homogenized at 0–4°C in 3 vol. of 20mM-Tris/HCl buffer, pH 7.5, containing 100mM-KCl, 5mM-MgSO₄, 1mM-EDTA, 0.2mM-fructose 1,6-bisphosphate and 10mM-2-mercaptoethanol. The homogenates were centrifuged at 10000g for 20min and then at 100000g for 60min, and the supernatant was used for assay of pyruvate kinase activity and to determine the quantity of the enzyme by a specific radioimmunoassay. Pieces of liver were also kept for determination of hepatic glycogen (Huijing, 1970).

Enzyme assay

Liver pyruvate kinase activity was assayed in a coupled enzymic reaction in 100mM-Tris/HCl buffer, pH 7.4, containing 100mM-KCl, 4mM-MgCl₂, 1mM-fructose 1,6-bisphosphate, 0.15mM-NADH, 2mM-ADP and 5 units of lactate dehydrogenase (Ishibashi & Cottam, 1978). The assay was initiated by the addition of 1mM-phosphoenolpyruvate and performed at 27°C. One unit of pyruvate kinase activity converts 1μmol of phosphoenolpyruvate into pyruvate/min.

Protein in the 100000g supernatant was determined by the colorimetric method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Radioimmunoassay

Initially, liver pyruvate kinase was purified by the procedure of Harada *et al.* (1978) up to the phosphocellulose ion-exchange chromatography step. It was further purified by adsorption on and elution from an Affi-Gel Blue (Bio-Rad Laboratories) column (Hall *et al.*, 1979). Enzyme purity was determined on 10%-polyacrylamide disc gels containing 4M-urea and 0.1% sodium dodecyl sulphate (Mizioro *et al.*, 1974). Homogeneous liver pyru-

vate kinase (100μg) was labelled with ¹²⁵I by the method of Winkelhake *et al.* (1979).

Anti-(liver pyruvate kinase) serum, generated in the goat, was kindly given by Dr. G. Larry Cottam (University of Texas Health Science Center, Dallas, TX, U.S.A.). Antibody specificity was determined on Ouchterlony double-diffusion plates prepared with 0.8% agar in 0.1M-potassium phosphate buffer, pH 7.2. The ability of the antibody to inhibit the activity of cytosolic and purified liver pyruvate kinase was also determined. It has previously been demonstrated that the phosphorylated and dephosphorylated forms of liver pyruvate kinase are equally reactive to the antibody (Riou *et al.*, 1978). The antiserum was diluted 1:25 with 10mM-Tris/HCl buffer, pH 7.0, containing 1mM-CaCl₂ and used in the specific radioimmunoassay of the enzyme.

The radioimmunoassay was done essentially as reported by Thompson & Drake (1982), except that 50μl of 6% (w/v) bovine serum albumin (fraction V) was added to each tube to decrease non-specific binding. Maximum binding of ¹²⁵I-labelled liver pyruvate kinase was obtained from 50μl of the diluted antiserum as determined by quantitative immunoprecipitation. A standard curve was obtained by competing ¹²⁵I-labelled liver pyruvate kinase with known amounts of the unlabelled homogeneous enzyme (0–8.0μg). The quantity of pyruvate kinase in livers from experimental animals was determined by adding various amounts of 100000g-supernatant protein (0.1–1.2mg) to the assay system.

Results

The diabetic rats used in this study lost 4.4 ± 14.4 g/week (mean $\pm$ s.d., $n = 33$) and had plasma glucose concentrations of 459 ± 67 mg/dl. In comparison, untreated rats gain 42.5 ± 5.6 g/week and had plasma glucose concentrations of 119 ± 16 mg/dl.

Insulin treatment of the diabetic rats had profound effects on liver size and hepatic glycogen content (Table 1). The liver weight increased steadily over the 60h period of insulin treatment, but there was no statistically significant change in body weight. This resulted in a 2.2-fold increase in liver weight/100g body wt. by 60h after insulin treatment. By comparison, liver glycogen content increased more rapidly, with maximum values of approx. 7% reached by 12h after insulin and maintained throughout the 60h treatment period. Treatment with the high-carbohydrate fat-free diet alone for 60h had a small effect on liver glycogen content and no effect on body weight, liver weight or liver weight/100g body wt.

Insulin treatment also mediated a substantial, though delayed, increase in liver pyruvate kinase

Table 1. *Body weights, liver weights and hepatic glycogen contents of experimental animals*

Rats, made diabetic with alloxan, were treated with insulin and a high-carbohydrate fat-free diet for the times indicated. Insulin treatment consisted of 4 units each of regular insulin and NPH insulin every 12 h. The number of rats in each group is given in parenthesis. Results are presented as means $\pm$ s.d. Statistical analysis compared the values from treated rats with the values from diabetic rats by Student's *t* test: **P* < 0.001; ***P* < 0.01.

	Body wt. (g)	Liver wt.		Hepatic glycogen (%, w/w)
		(g)	(g/100 g body wt.)	
Diabetics (5)	164 $\pm$ 20	8.5 $\pm$ 1.3	5.3 $\pm$ 0.6	1.37 $\pm$ 0.43
Insulin-treated				
12h (6)	161 $\pm$ 20	12.5 $\pm$ 2.0**	7.8 $\pm$ 0.7*	6.86 $\pm$ 0.42*
36h (7)	167 $\pm$ 25	17.5 $\pm$ 2.7*	10.6 $\pm$ 1.3*	7.71 $\pm$ 0.79*
60h (7)	192 $\pm$ 25	21.8 $\pm$ 1.3*	11.5 $\pm$ 1.5*	6.61 $\pm$ 0.27*
Diabetic on special diet; 60h (8)	169 $\pm$ 36	7.6 $\pm$ 1.8	4.5 $\pm$ 0.2	2.48 $\pm$ 0.31*

Table 2. *Insulin effect on liver pyruvate kinase activity*

Livers were removed and homogenized in 3 vol. of 20 mM-Tris/HCl buffer, pH 7.5, containing 100 mM-KCl, 5 mM-MgSO₄, 1 mM-EDTA, 0.2 mM-fructose 1,6-bisphosphate and 10 mM-2-mercaptoethanol. The homogenates were centrifuged, and the 100000 g supernatants were assayed for pyruvate kinase activity. The number of rats in each group is given in parenthesis. Results are presented as means $\pm$ s.d. Statistical analysis compared the values from treated rats with the values from diabetic rats by Student's *t* test: **P* < 0.001.

	Activity		
	(units/mg of supernatant protein)	(units/g of liver)	(units/g body wt.)
Diabetic (5)	0.077 $\pm$ 0.018	4.41 $\pm$ 0.49	0.23 $\pm$ 0.04
Insulin-treated			
12h (6)	0.061 $\pm$ 0.042	3.86 $\pm$ 1.00	0.30 $\pm$ 0.10
36h (7)	0.184 $\pm$ 0.051*	8.36 $\pm$ 2.44*	0.88 $\pm$ 0.28*
60h (7)	0.502 $\pm$ 0.110*	17.99 $\pm$ 4.41*	1.99 $\pm$ 0.44*
Diabetic on special diet; 60h (8)	0.064 $\pm$ 0.011	3.63 $\pm$ 1.23	0.16 $\pm$ 0.06

Table 3. *Quantity of liver pyruvate kinase*

Total liver pyruvate kinase (nmol) was determined by specific radioimmunoassay in the different experimental groups. The number of rats in each group is given in parenthesis. Results are presented as means $\pm$ s.d. Statistical analysis was based on a comparison by Student's *t* test: **P* < 0.001; ***P* < 0.01.

	Amount of liver pyruvate kinase		Activity of liver pyruvate kinase (units/nmol of enzyme)
	(nmol/100 g body wt.)	(nmol/g of liver)	
Diabetic on special diet; 60h (8)	1.24 $\pm$ 0.59	0.28 $\pm$ 0.13	15.42 $\pm$ 8.86
Insulin-treated 60h (7)	7.17 $\pm$ 1.10*	0.64 $\pm$ 0.15*	28.85 $\pm$ 7.34**

activity (Table 2). The pyruvate kinase activity (units/g body wt.) showed no response in the first 12 h of insulin treatment, a significant increase by 36 h, and a more substantial increase by 60 h after insulin. Diabetic rats maintained on the high-carbohydrate fat-free diet for 60 h without insulin showed no increase in enzyme activity. Expressing the data as either units/mg of supernatant protein or units/g of liver demonstrated the same pattern of enzyme stimulation (Table 2).

A specific radioimmunoassay method was used to determine whether the increase in liver pyruvate

kinase activity was the result of an increase in enzyme quantity. The sensitivity of the specific radioimmunoassay was determined by competing ¹²⁵I-labelled liver pyruvate kinase against known amounts of the unlabelled homogeneous enzyme. The radioimmunoassay was sensitive to as little as 250 ng of unlabelled enzyme, with 50% displacement of ¹²⁵I-labelled pyruvate kinase obtained with 2.0 μ g of unlabelled antigen.

The results of the radioimmunoassay (Table 3) demonstrated that the quantity of pyruvate kinase (nmol/100 g body wt.) increased 5.8-fold after

insulin treatment for 60h, and that the amount of the enzyme/g of liver increased 2.3-fold. These results indicate that the quantity of pyruvate kinase increased in excess of the amount that could be attributed to the increased liver size. Furthermore, the radioimmunoassay showed a moderate increase in the specific activity (units/nmol) of the enzyme.

Discussion

The method of induction of experimental diabetes in this study, i.e. intravenous injection of 4% alloxan at a dosage of 55mg/kg body wt., produced an insulin-deficient condition coupled with hyperglycaemia and weight loss in most rats. However, some rats became severely hyperglycaemic, yet continued to gain moderate to substantial amounts of weight. Because of this variability in weight gain, classification of a rat as diabetic required strict adherence to the criteria first proposed by Lazarow & Palay (1946). They suggested that diabetic rats should have blood glucose concentrations in excess of 300mg/dl and little or no weight gain. These requirements are indicative of the metabolic defects observed in chronic diabetes. Hyperglycaemia results from an impairment of both intracellular glucose utilization and, in non-hepatic tissue, glucose transport (Steiner, 1966). Weight loss results from the general catabolic effects of diabetes leading to decreased protein synthesis and wasting of adipose tissue (Pilkis & Park, 1974). To assure that the rats met these criteria they were maintained for 10 days before use.

The insulin-mediated increase in liver pyruvate kinase activity observed in the present study (Table 2) was similar to that reported by Chang & Schneider (1971). However, it differs significantly from several other reports. An earlier report by Tanaka *et al.* (1967) demonstrated an insulin stimulation of liver pyruvate kinase activity by 6h in diabetic rats, whereas Weber *et al.* (1965) observed an increase by 24h after insulin. We observed no stimulation at these or earlier time points. The differences in these studies may be due to the variability in the diabetic model. Chang & Schneider (1971) used animals that were diabetic for at least 8 weeks and showed significant weight loss before the initiation of insulin treatment. This diabetic model, being comparable with ours, may account for the similarity of results. In contrast, Weber *et al.* (1965) and Tanaka *et al.* (1967), using animals 7 and 5 days after alloxan treatment respectively, supplied no information on the body weights of these diabetic animals. This information is of considerable importance in assessing the severity of the diabetic condition, as discussed above. Without these body-weight data it is difficult to explain the differences in the kinetics of the insulin-stimulated induction of liver

pyruvate kinase activity presented in the two earlier papers.

It is possible that the delayed rise in liver pyruvate kinase activity observed in our present study was due to an inability of our diabetic rats to respond properly to insulin. However, this is unlikely, since insulin stimulation of glycogenesis was clearly evident at 12h after insulin. In fact, hepatic glycogen content has been shown to increase significantly as early as 1h after insulin in similarly prepared alloxan-induced diabetic rats (Thompson *et al.*, 1981). Additionally, an insulin-mediated increase in liver weight (Table 1) occurred rapidly, which is consistent with previous observations (Osborn *et al.*, 1953). Therefore the delayed stimulation of liver pyruvate kinase activity (Table 2) cannot be attributed to an inability of the livers from these alloxan-treated diabetic rats to respond to insulin.

The mechanism controlling this insulin-mediated increase in liver pyruvate kinase activity had to involve an increase in either the quantity or the specific activity of the enzyme. The radioimmunoassay data given in the present paper conclusively demonstrate that both events had occurred (Table 3), although the increase in enzyme quantity appeared to be more significant than the enhancement in specific activity.

Contrary to these findings are the results reported by Johnson & Veneziale (1980). They report that liver pyruvate kinase activity is increased in insulin-treated diabetic rabbits owing to a change in the specific activity (units/nmol) of the enzyme. There was no alteration in enzyme quantity. This difference in results could be attributed to the diabetic model used in their study. Their animals, which began receiving insulin 72h after alloxan, would have been diabetic for only 24–48h, since constant hyperglycaemia after alloxan treatment is not reached until 24–48h after administration of the drug (Rerup, 1970). Therefore, since the half-life of liver pyruvate kinase is approx. 55h (Hopkirk & Bloxham, 1979; Cladaras & Cottam, 1980), a sufficient quantity of the enzyme molecules probably still exist that, after modification to increase the catalytic activity of each enzyme molecule, would support the demand for increased carbohydrate utilization. In our chronically diabetic rats the quantity of liver pyruvate kinase was severely depressed, necessitating an increase in both enzyme quantity and specific activity to handle the hormonally induced increase in carbohydrate utilization.

In conclusion, insulin mediated a substantial increase in the activity of liver pyruvate kinase in chronically diabetic rats after an initial delay of 12h. Furthermore, this increase in pyruvate kinase activity was attributed to a dual mechanism consisting of a substantial increase in the quantity of the enzyme and a moderate increase in its specific activity. A mechanism that first involved enzyme synthesis

de novo, e.g. as observed in the dietary induction of liver pyruvate kinase (Cladaras & Cottam, 1980), followed by a modification of the newly synthesized enzyme molecules, would be entirely consistent with our results.

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