Effects of Chronic Hyperinsulinaemia on Hepatic Enzymes Involved in Lipogenesis and Carbohydrate Metabolism in the Young Rat

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Chronic (6 days) hyperinsulinaemia in young rats produced lower blood glucose concentrations and augmented body- and liver-weight gain. The insulin-treated rats had increased hepatic activities of citrate-cleavage enzyme, 'malic' enzyme and high-substrate (6.6 mM-phosphoenolpyruvate) pyruvate kinase, and decreased glucose 6-phosphatase. There were no changes in activities of phosphoenolpyruvate carboxykinase, phosphofructokinase, low-substrate (1.3 mM-phosphoenolpyruvate) pyruvate kinase, glucokinase and hexokinase.

The effects of acute insulin administration or deprivation *in vivo* or *in vitro* on hepatic enzyme activities have been studied extensively (Weber *et al.*, 1965, 1966; Clark & Lardy, 1975). Insulin given acutely to either alloxan-treated hyperglycaemic diabetic rats or perfused isolated normal liver resulted in increased hepatic enzyme activities of pyruvate kinase (EC 2.7.1.40) (Taunton *et al.*, 1972; Blair *et al.*, 1976), glucokinase (EC 2.7.1.2) (Salas *et al.*, 1963; Sharma *et al.*, 1963), phosphofructokinase (EC 2.7.1.11) (Taunton *et al.*, 1972), 'malic' enzyme (EC 1.1.1.40) (Shrago *et al.*, 1963) and citrate-cleavage enzyme (EC 4.1.3.8) (Söling *et al.*, 1969), and decreased activities of phosphoenolpyruvate carboxykinase (EC 4.1.1.32) (Shrago *et al.*, 1963) and glucose 6-phosphatase (EC 3.1.3.9) (Langdon & Weakley, 1955).

The purpose of the present investigation was to determine the effect of continuous 6-day hyperinsulinaemia on the hepatic activities of these enzymes, which modify glucose and fatty acid metabolism, in the intact non-alloxan-treated young rat. A subcutaneously implanted osmotically driven mini-pump was utilized for continuous hormone delivery.

Experimental

Male Charles River rats initially weighing 87–118 g were fed with standard laboratory chow *ad libitum* and had free access to 10% (w/v) glucose as fluid intake; after preliminary observations, the latter was

substituted for water to mitigate the mortality after hyperinsulinaemia. Regular pig insulin, U-500 ILETIN (Eli Lilly Co., Indianapolis, IN, U.S.A.), was administered by means of a 2.2 cm × 0.5 cm osmotically driven minipump, ALZET (ALZA Corp., Palo Alto, CA, U.S.A.), designed to deliver *in vivo* 0.85 μ l/h at a constant rate over 1 week, implanted subcutaneously in the animal's back. Control rats underwent 5 min sham surgical procedures, including injection of 2.5 mg of ketamine intraperitoneally and 2 mg of lidocaine intradermally as anaesthesia. The experimental groups were infused with either 9.2 units (25 units = 1 mg of insulin) or 4.6 units of insulin/day. Blood samples were obtained from non-starved animals without anaesthesia by nicking a tail vein after dilation in lukewarm water, and were taken at intervals of at least 48 h between 09:30 and 11:00 h on each occasion.

Plasma glucose was determined by a glucose oxidase reagent from Boehringer Mannheim Corp. (Indianapolis, IN, U.S.A.) and insulin by a double-antibody radioimmunoassay reagent from Amersham Corp. (Arlington Heights, IL, U.S.A.). Glucose assay varied by $\pm 2\%$, and the insulin assay varied between $\pm 2\%$ at 10 μ units/ml and $\pm 5\%$ at 80 μ units/ml.

The animals were killed by decapitation, and the livers immediately removed, chilled on ice, weighed and stored at -70°C pending enzyme analysis. All homogenates were prepared at $0-4^{\circ}\text{C}$ with a Potter–Elvehjem power-driven homogenizer in appropriate media, depending on the assay.

For citrate-cleavage enzyme and phosphoenolpyruvate carboxykinase, 20 mM-Tris/0.25 M-sucrose/10 mM-2-mercaptoethanol, adjusted with HCl to pH 8.0, was used to make a 7:1 (v/w) liver homo-

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Table 1. *Rat biochemical and growth profiles during 6 days of hyperinsulinaemia*

Continuous insulin administration was performed as described in the Experimental section. Insulin and glucose values represent three separate determinations in temporal sequence, at least 48 h apart, after insulin administration had commenced. Abbreviation: N.S., not significant, $P > 0.05$.

Insulin dosage (units/day)	Rat no.	Plasma insulin (μ units/ml)	Plasma glucose (mm)	Body-weight gain (g/day)	Liver wt. (% of body wt.)
9.2	1	2150, 1150, 1500	2.61, 1.72, 4.61	12.5	5.3
	2	2150, 1750, 975	4.61, 5.72, 1.83	14.0	5.2
	3	1350, 1450, 1300	1.83, 4.33, 2.16	13.0	5.1
	4	450, 1300, 1400	1.67, 2.00, 2.55	13.8	4.6
	5	1850, 1425, 875	1.67, 2.78, 1.67	11.8	5.4
	6	1375, 1450, 1850	2.66, 2.33, 2.50	12.2	5.3
Mean $\pm$ S.E.M.		1431 $\pm$ 101	2.72 $\pm$ 0.28	12.0 $\pm$ 0.4	5.2 $\pm$ 0.1
4.6	7	525, 145, 400	2.00, 2.28, 3.89	14.7	5.1
	8	950, 575, 1150	1.61, 2.05, 4.22	12.7	4.8
	9	85, 55, 90*	4.55, 7.16, 7.83	7.3	5.0
	10	400, 385, 800	2.33, 2.16, 4.66	11.8	4.7
	11	1000, 440, 380	2.39, 2.66, 3.61	12.7	5.1
	12	100, 100, 55*	3.00, 7.44, 4.61	5.7	4.4
	13	460, 95, 750	4.00, 3.83, 2.33	9.7	5.3
Mean $\pm$ S.E.M.		426 $\pm$ 74	3.72 $\pm$ 0.39	10.7 $\pm$ 1.2	4.9 $\pm$ 0.1
0 (control)	14	34, 28, 30	5.66, 6.33, 7.22	7.0	4.9
	15	38, 22, 24	5.55, 6.05, 6.72	6.0	5.0
	16	30, 30, 30	5.55, 6.72, 6.83	7.0	4.5
	17	34, 30, 28	6.88, 6.72, 7.66	5.7	4.5
	18	46, 30, 28	7.22, 7.55, 7.38	7.7	4.7
	19	38, 34, 32	6.33, 6.61, 6.99	8.7	4.5
	20	28, 26, 22	6.83, 7.33, 6.38	5.5	4.5
Mean $\pm$ S.E.M.		31 $\pm$ 1	6.72 $\pm$ 0.11	6.8 $\pm$ 0.4	4.7 $\pm$ 0.1
9.2 units/day versus control		$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.005$
4.6 units/day versus control		$P < 0.005$	$P < 0.001$	$P < 0.025$	N.S.

* Presumed technical failure of insulin delivery.

genate. For pyruvate kinase and phosphofructokinase, a 10:1 (v/w) homogenate was prepared with 20mM-Tris/5mM-MgSO₄/120mM-KCl/0.1mM-EDTA, adjusted with HCl to pH 7.5. For 'malic' enzyme, a 3:1 (v/w) homogenate was prepared in 0.25M-sucrose, and, for glucokinase and hexokinase, a 4:1 (v/w) homogenate was prepared in 0.15M-KCl/5mM-EDTA/10mM-2-mercaptoethanol/4mM-MgSO₄ at pH 7.2. For glucose 6-phosphatase, a 2:1 (v/w) homogenate was prepared with 0.25M-sucrose and 1mM-EDTA. All homogenates were centrifuged for 1 h at 4°C at 105000g in a refrigerated ultracentrifuge, except for glucose 6-phosphatase, for which the homogenate was centrifuged at 10000g for 30min.

Standard spectrophotometric assays were used without modification for glucokinase and hexokinase (Sharma *et al.*, 1963), phosphofructokinase (Underwood & Newsholme, 1965), citrate-cleavage enzyme (Srere, 1959) and 'malic' enzyme (Hsu & Lardy,

1969). Pyruvate kinase was determined by the method of Blair *et al.* (1976) at two phosphoenolpyruvate concentrations, one subsaturating at 1.3 mM, the other saturating at 6.6 mM. Glucose 6-phosphatase was assayed as described by Baginski *et al.* (1974), but P_i was determined by molybdovanadophosphoric acid extraction in butanol (Parvin & Smith, 1969). Phosphoenolpyruvate carboxykinase was assayed by a modification of the method of Seubert & Huth (1965). The reaction mixture consisted of 50mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/2mM-ITP/2mM-MgCl₂/1mM-reduced glutathione/1.5mM-oxalacetate adjusted to pH 7.5 in a total volume of 1 ml. The reaction was terminated after 5 min at 25°C by the addition of 12mg of tetraethylammonium tetrahydridoborate in 0.1 ml of 7.5mM-NaOH.

All enzyme analyses were conducted in duplicate at 25°C, except for glucose 6-phosphatase, which was

Table 2. *Hepatic enzyme activities after 6 days of continuous hyperinsulinaemia*

Insulin administration was performed as described in the Experimental section. Values represent means $\pm$ S.E.M. expressed in units. The number of animals in each group is listed in parenthesis. Abbreviation: N.S. not significant, $P > 0.05$.

Insulin dose (units/day)	'Malic' enzyme	Citrate-cleavage enzyme	Glucose-kinase	Hexokinase	Phospho-fructo-kinase	Glucose 6-phosphatase	Phospho-phenol-pyruvate carboxy-kinase	Pyruvate kinase	
								1.3 mM-substrate	6.6 mM-substrate
9.2 (6)	6.42 $\pm$ 0.56	2.14 $\pm$ 0.38	0.41 $\pm$ 0.03	0.27 $\pm$ 0.02	0.62 $\pm$ 0.09	6.28 $\pm$ 0.42	1.59 $\pm$ 0.44	7.10 $\pm$ 1.39	54.0 $\pm$ 3.2
4.6 (7)	4.53 $\pm$ 0.56	1.21 $\pm$ 0.16	0.33 $\pm$ 0.03	0.26 $\pm$ 0.01	0.67 $\pm$ 0.14	6.00 $\pm$ 0.81	1.72 $\pm$ 0.43	9.60 $\pm$ 0.65	43.7 $\pm$ 3.4
0 (7)	4.29 $\pm$ 0.35	0.61 $\pm$ 0.09	0.35 $\pm$ 0.04	0.31 $\pm$ 0.01	0.71 $\pm$ 0.14	9.38 $\pm$ 0.71	1.70 $\pm$ 0.40	7.11 $\pm$ 1.53	35.4 $\pm$ 5.1
9.2 units/day versus control	$P < 0.01$	$P < 0.005$	N.S.	N.S.	N.S.	$P < 0.005$	N.S.	N.S.	$P < 0.025$
4.6 units/day versus control	N.S.	$P < 0.01$	N.S.	N.S.	N.S.	$P < 0.01$	N.S.	N.S.	N.S.

assayed at 37°C. For all enzymes assayed, 1 unit of activity is defined as 1 μ mol of substrate transformed/min per g wet wt. of liver. Statistical analysis was performed by using unpaired Student's *t* tests and linear-regression analysis.

Results

Rats receiving insulin (9.2 units daily) by the continuous subcutaneous route for 6 days had mean plasma insulin concentrations of 1431 μ units/ml compared with control animals whose plasma insulin concentrations were 31 μ units/ml ($P < 0.001$) (Table 1). Rats treated with 4.6 units/day had plasma insulin concentrations of 942 μ units/ml ($P < 0.005$ versus control). The former group gained weight at 12.0 g/day compared with the controls' 6.8 g/day ($P < 0.001$); the latter group gained 10.7 g/day ($P < 0.025$ versus control). In addition, the blood glucose concentrations for both hyperinsulinaemic groups were lower ($P < 0.001$) than in the untreated controls. Liver weight, expressed as percentage of body weight, was higher only for the group receiving 9.2 units/day ($P < 0.005$).

The hyperinsulinaemic rats given 9.2 units/day showed significantly elevated activities of 'malic' enzyme ($P < 0.01$), citrate-cleavage enzyme ($P < 0.005$) and high-substrate (6.6 mM-phosphoenolpyruvate) pyruvate kinase ($P < 0.025$), and decreased glucose 6-phosphatase ($P < 0.005$) enzyme activity compared with controls (Table 2). However, the changes in enzyme activities in rats given 4.6 units/day were significant ($P < 0.01$) only for elevated citrate-cleavage enzyme activity and decreased glucose 6-phosphatase activity compared with controls.

Linear-regression analysis of enzyme activity in units versus mean plasma insulin concentration for each individual was significant for citrate cleavage enzyme ($P < 0.001$, $\pi = 0.72$), 'malic' enzyme ($P < 0.001$, $\pi = 0.73$), high-substrate phosphoenolpyruvate pyruvate kinase ($P < 0.001$, $\pi = 0.71$) and glucose 6-phosphatase ($P < 0.02$, $\pi = 0.54$); only the last

enzyme activity versus mean plasma insulin concentration was not significant if the controls were excluded from the analysis.

The activities of phosphofructokinase, glucokinase, hexokinase, low-substrate (1.3 mM-phosphoenolpyruvate) pyruvate kinase and phosphoenolpyruvate carboxykinase in the hyperinsulinaemic animals did not differ significantly from those of controls.

Discussion

Insulin is a well-known lipogenic hormone and would be expected to increase the activities of those enzymes involved in fatty acid synthesis. Both 'malic' enzyme, which generates NADPH, a necessary substrate for fat synthesis, and citrate-cleavage enzyme, which utilizes cytosolic citrate as a precursor for fatty acid synthesis (Srere & Bhaduri, 1962), have been shown to be hormone-responsive (Söling *et al.*, 1969). Our chronically hyperinsulinaemic rats displayed increased hepatic activities of citrate cleavage enzyme and 'malic' enzyme, directly proportional to their mean plasma insulin concentrations, consistent with other observations after acute insulin administration (Shrago *et al.*, 1963; Young *et al.*, 1964). Shrago *et al.* (1963) administered identical daily injections of 20 units of protamine zinc insulin to both normal and alloxan-treated adult diabetic rats; the former group had a 2½-fold increase in hepatic 'malic' enzyme activity compared with a 20-fold increase in the hyperglycaemic rats. Citrate-cleavage enzyme was shown to increase by nearly 500% after repeated insulin injections (2 units of insulin every 2 h the day before death and 6 units of insulin zinc suspension the night before) in alloxan-diabetic rats (Söling *et al.*, 1969).

Sladek & Snarr (1974), using [14 C]alanine, reported inhibition of hepatic gluconeogenesis in starved rats by insulin in the isolated perfused liver *in situ*. Both groups of hyperinsulinaemic animals in our model had decreased hepatic activities of the gluconeogenic enzyme glucose 6-phosphatase. Lang-

don & Weakley (1955) reported that acute administration of 200 units of protamine zinc insulin/kg to diabetic rats 12 h before death produced a profound decrease in enzyme activity, to nearly normal pre-alloxan-treatment values. Ruderman & Lauris (1968) showed an increase (1.1-fold) in this enzyme 3 h after the administration of anti-insulin serum compared with rats receiving only normal serum.

The other gluconeogenic enzyme assayed, phosphoenolpyruvate carboxykinase, did not decrease in out chronically hyperinsulinaemic animals, in contrast with the effects of acute insulin administration shown by Shrago *et al.* (1963). They reported a 5-fold decrease in phosphoenolpyruvate carboxykinase activity after 3 days of daily injections of 20 units of protamine zinc insulin in alloxan-diabetic rats; the identical insulin dose administered to normal non-hyperglycaemic rats produced a slight non-significant increase in activity.

Blair *et al.* (1976) studied the regulation of pyruvate kinase activity in perfused normal rat liver at two different phosphoenolpyruvate substrate concentrations, subsaturating (1.3 mM) and saturating (6.6 mM). They proposed that glucagon and insulin regulate the interconversion of two kinetically distinct forms of pyruvate kinase. Insulin (22 munits/ml) or glucagon (0.14 μ M) alone in the perfusate did not alter the pyruvate kinase activity at saturating 6.6 mM-phosphoenolpyruvate; however, the former hormone did significantly ($P < 0.05$) increase the pyruvate kinase activity at subsaturating 1.3 mM-phosphoenolpyruvate in livers of fed rats. Likewise, Taunton *et al.* (1972) reported a more than 2-fold rise in hepatic pyruvate kinase activity (at subsaturating phosphoenolpyruvate concentration of 0.78 mM) within 15 min after intravenous injection of 0.15 unit of insulin/kg in the anaesthetized rat. We did not find any difference in pyruvate kinase at subsaturating 1.3 mM-phosphoenolpyruvate in the chronically hyperinsulinaemic rats compared with controls, but did find that the activity of pyruvate kinase at saturating 6.6 mM-phosphoenolpyruvate was significantly ($P < 0.001$) related to the mean plasma insulin concentration.

Other key glycolytic enzymes, glucokinase and phosphofructokinase, did not change significantly in the chronically hyperinsulinaemic animals, a finding in contrast with the increased activities after acute exposure to insulin (Sharma *et al.*, 1963; Ruderman & Lauris, 1968; Taunton *et al.*, 1972). Taunton *et al.* (1972) demonstrated more than a 1½-fold increase in phosphofructokinase within 15 min in the intact anaesthetized animal after intravenous injection of 0.15 unit of insulin/kg. Hepatic glucokinase activity was shown by Salas *et al.* (1963) to increase from less than 0.05 to 0.93 unit after 32 h of repeated insulin injections in alloxan-diabetic rats. When anti-insulin serum was adminis-

tered to normal rats, there was a 30% decrease in glucokinase activity within 3 h compared with controls (Ruderman & Lauris, 1968). Hexokinase was not altered in our hyperinsulinaemic groups, consistent with the effects of insulin treatment in alloxan-diabetic rats (Salas *et al.*, 1963; Sharma *et al.*, 1963).

Some discrepancies between the effects of acute and chronic insulin administration on hepatic enzyme activities might be secondary to alloxan, dietary manipulations, such as 10% glucose for oral fluid intake, or other hormonal effects (glucagon, adrenaline, growth hormone etc.) resulting from the hyperinsulinaemic state and attendant lower plasma glucose concentrations. To date, no well-controlled studies have investigated the possible independent action of the cytotoxic agent alloxan on hepatic enzyme responsiveness to hormonal and metabolic alterations. Jakobsson & Dallner (1968) reported an increased K_m and V_{max} in membrane-bound glucose 6-phosphatase after microsomal incubation *in vitro* with 0.025% alloxan, and concluded that the initial rise (about 5 h) in this enzyme activity after induction of alloxan-diabetes is a direct result of the drug. Insulin effects on alloxan-exposed livers may be significantly different from the hormonal effects on isolated or non-drug-exposed organs. By utilizing a surgically implanted osmotic mini-pump producing continuous hyperinsulinaemia, the use of alloxan and the trauma resulting from daily injections has been avoided.

The present study indicates that chronic hyperinsulinaemia, with concomitant lower blood glucose concentrations, augmented body-weight gain and increased liver weight, produced increased hepatic activities of 'malic' enzyme, citrate-cleavage enzyme and pyruvate kinase at saturating phosphoenolpyruvate concentrations, and decreased glucose 6-phosphatase, but did not appear to alter the assayable activities of phosphoenolpyruvate carboxykinase, phosphofructokinase, glucokinase, hexokinase or pyruvate kinase at subsaturating phosphoenolpyruvate concentrations.

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