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INSULIN DEGRADATION

XXV. GLUTATHIONE-INSULIN TRANSHYDROGENASE ACTIVITY OF RAT LIVER AND KIDNEY DURING THE DEVELOPMENT OF STREPTOZOTOCIN-DIABETES

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Summary

The activity of glutathione-insulin transhydrogenase (glutathione:protein-disulfide oxidoreductase, EC 1.8.4.2) in the liver and kidneys of rats during the development of streptozotocin-induced diabetes has been studied. Following a single injection of streptozotocin, the transhydrogenase activity fell rapidly for 7-8 days and then gradually with time in both organs. In contrast to the control rats where approximately 25% of the enzyme is in a 'latent' state, nearly all the transhydrogenase activity in the diabetic liver appears to be in the free or functional form. The results are consistent with the hypothesis that both hepatic and renal glutathione-insulin transhydrogenase activity are under feedback control by circulating insulin. The possibility is discussed that the latent state may represent a storage form of the enzyme, which in insulin-insufficiency states is mobilized to the free or functional form for cell function.

Introduction

Glutathione-insulin transhydrogenase (glutathione:protein-disulfide oxidoreductase, EC 1.8.4.2) is an ubiquitous enzyme. It inactivates insulin by catalyzing the reduction of the disulfide bonds in the presence of a thiol such as glutathione, thereby releasing the A and B chains of insulin. The cleavage of

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insulin by transhydrogenase is the first and rate-controlling step in the sequential pathway of insulin metabolism, both at physiological and pharmacological concentrations of the hormone. The possibility has been suggested that transhydrogenase may function to mediate insulin action. (See Refs. 1 2 for recent reviews and references.)

When rats are made diabetic either by the injection of alloxan or streptozotocin, the activity of hepatic transhydrogenase is significantly lowered [3—7]. Insulin treatment of diabetic animals restores the hepatic transhydrogenase activity to normal or above normal levels [3—7]. Similarly, when rats are fasted for periods of up to 65 h, the activity of transhydrogenase is decreased but is restored on refeeding [3—5]. These results are consistent with the hypothesis that circulating insulin acts as an inducer of liver GSH-insulin transhydrogenase. Such control by insulin of the activity of the enzyme could serve as part of an important feedback loop for regulating the amount of insulin available to the systemic circulation.

Studies with the obese hyperglycemic mouse (*ob/ob*), a genetic mutant characterized by a marked hyperinsulinemia, hyperglycemia and resistance to the hypoglycermic action of insulin, showed that hepatic GSH-insulin transhydrogenase activity is markedly higher than in lean litter mates [8], again supporting the proposal of feedback control by insulin over the enzyme. Furthermore a major portion of the increased hepatic transhydrogenase in the *ob/ob* mouse occurs in a latent state; i.e., the increased amount of enzyme appears to be either unavailable or it is non-functional (see Refs. 1, 8, 9 and 10 for further discussion on latent and non-latent forms of the enzyme). Nevertheless the *ob/ob* mouse liver still contains more of the functional form of transhydrogenase than that of the lean mouse [8].

All studies cited above were performed using animals in chronic conditions (i.e., prolonged starvation, overt diabetes, obesity, etc.). In the present study, we have followed the alteration in the activity of GSH-insulin transhydrogenase in the liver and kidneys of rats with the development of streptozotocin-diabetes. In view of the previous findings of a higher degree of latency in the *ob/ob* mouse, i.e., hyperglycemia and hyperinsulinemia, [8], we have also examined the effect of diabetes, i.e., hyperglycemia and hypoinsulinemia, on the latency of the hepatic enzyme.

Materials and Methods

Male Holtzman rats (Rolfsmeyer Laboratory Animals, Springfield, OH), weighing 180—210 g at the start of the experiment, were used. The rats were caged individually in a room maintained at 23°C and where the lights were alternately on and off for 12 h periods. The rats were allowed free access to food and water. They were randomly divided into two groups and 36 rats from one group were rendered diabetic by the intravenous injection of streptozotocin (Upjohn, Kalamazoo, MI) at a concentration of 60 mg/kg body weight. The streptozotocin was dissolved in 0.1 M sodium citrate buffer, pH 4.5, and the solution (0.2 ml) injected into the tail vein under light ether anesthesia. The control animals were injected with the sodium citrate buffer. Glucosuria was detected in all the rats injected with streptozotocin within 48 h and was monitored with Clinistix (Ames Co.).

Groups of three or four animals were investigated 1, 3, 7, 10, 17, 24, 31 and 38 days after the induction of diabetes and compared with a control group of normal animals.

The rats were killed by decapitation, blood was collected and the serum and plasma were separated for insulin and glucose measurements, respectively. Insulin concentrations were determined by a double-antibody immunoassay procedure [18] with rat insulin (Novo Co., Copenhagen, Denmark) as a standard. Glucose was measured by a glucose oxidase-peroxidase colorimetric method (Boehringer Mannheim Corporation, New York).

Livers and kidneys were excised, rinsed with ice-cold water, blotted free of moisture and weighed. The livers were immediately homogenized but the kidneys were stored at −20°C until required. A 20 percent tissue homogenate was prepared by mixing a 1 g piece of tissue with 4 ml of 0.25 M sucrose/5 mM EDTA buffered to pH 7.5 with 50 mM Tris and homogenized using a Teflon-fitted Potter-Elvejhem homogenizer [10]. The tissue homogenate thus obtained was used for the assay of DNA content. The homogenates were centrifuged at 5100 × *g* for 10 min at 4°C and at 10 000 × *g* for 15 min; the sediment was discarded after each centrifugation. The tissue supernatants thus obtained were used for the assay of free (i.e., non-latent) transhydrogenase activity (cf. Refs. 8 and 10) and protein content. In separate control experiments it was established that the recovery of the transhydrogenase activity in 10 000 × *g* supernatants from normal and diabetic liver homogenates was consistently around 70%. The values of the enzyme activity per unit DNA have not been corrected for the loss in the recovery of the enzyme activity since the relative values within the groups and between the normal and diabetic groups will remain unchanged.

In order to measure the total (i.e., latent plus free) activity of transhydrogenase present, the supernatants were homogenized for 40 s with the Polytron homogenizer at a rheostat setting of 10 as described previously [12].

Glutathione-insulin transhydrogenase was measured by the conversion of the radioactivity of [^{125}I]-labeled insulin to a form soluble in 5% trichloroacetic acid [8]. It has been demonstrated previously [8] that under the assay conditions described (i.e., in the presence of 1 mM GSH/5 mM EDTA), the insulin degradation observed is solely because of the action of transhydrogenase. The enzyme activities were measured at three or four concentrations of tissue protein. All enzyme activities reported are based on the protein concentrations yielding linear reaction rates and have been corrected for the non-enzymic degradation of insulin.

Protein was determined by the Lowry method [13] using bovine serum albumin (Sigma Co.) as a standard, and DNA by the diphenylamine reaction [14]. The preparation and purification of [^{125}I]iodoinsulin (approx. 0.6 atom of iodine/molecule) has been described previously [15].

Results

Activities of GSH-insulin transhydrogenase are presented as activity in terms of unit protein and unit DNA. As previously pointed out [4] expression of data in terms of DNA is probably the most accurate parameter. The results were however the same whether expressed in terms of protein or DNA.

The effects of a single injection of streptozotocin on the hepatic GSH-insulin transhydrogenase activity of rats are presented in Table I. A significant fall ($P < 0.05$) in free transhydrogenase activity when expressed in terms of DNA was seen 1 day after the injection. The activity fell rapidly during the first 7—8 days to 64 percent of that of the control animals. Then there was a gradual decrease of activity with time (24 days); thereafter, transhydrogenase activity apparently stabilized at about 42 percent that of the controls. The slight upward swing in the activity on day 38 was not significant and was not observed when the activity was based in terms of unit protein.

In previous experiments [10], it was observed that the transhydrogenase activity of rat liver homogenates prepared by a Polytron homogenizer is much greater than in the homogenates prepared with a Potter-Elvehjem homogenizer and this led to the discovery that a significant percentage of liver transhydrogenase is in a latent state. Several methods are available to estimate the proportion of transhydrogenase present in the latent state; these include homogenization with a Polytron homogenizer, sonication, freezing and thawing and treatment with the non-ionic detergent Triton X-100 [10]. The presence of Triton X-100 may modify the apparent K_m (insulin) of the enzyme [8] and as the mechanism of activation by Triton has not been delineated, a mechanical procedure, namely homogenization using a Polytron homogenizer, was employed in the present studies.

With rat liver from the control animals, use of the Polytron led to a 25 percent increase in tranhydrogenase activity; thus about 75% of the enzyme is in a 'functional' form. This is comparable to the figure of 71% found in the microsomal portion of liver from lean mice [8]. In the streptozotocin-diabetic

TABLE I

THE EFFECT OF A SINGLE INJECTION OF STREPTOZOTOCIN (60 mg/kg BODY WEIGHT) ON THE ACTIVITY OF GLUTATHIONE-INSULIN TRANSHYDROGENASE IN RAT LIVER

Each liver was homogenized in 0.25 M sucrose 5 mM EDTA/50 mM Tris buffer, pH 7.5, with a Teflon-fitted Potter-Elvejhem homogenizer and the homogenates centrifuged. A portion of the supernatant was used for the measurement of 'free' glutathione-insulin transhydrogenase activity and the remainder homogenized with a Polytron homogenizer as described in the text. The Polytron-treated supernatants were assayed for the 'total' glutathione-insulin dehydrogenase activity. The enzyme activities are expressed in units per mg of DNA and each value is the mean ± S.D. for three or four animals (shown in brackets). The *P* values were determined by the use of Student's *t*-test.

Time after injection (days)	Glutathione-insulin transhydrogenase activity					
	Control animals		Diabetic animals		*P* values	
	A 'Free' activity	B 'Total' activity	C 'Free' activity	D 'Total' activity	A—C	B—D
1	2.97 ± 0.19	4.02 ± 0.32 (4)	3.22 ± 0.12	3.54 ± 0.21 (4)	<0.05	<0.05
3	2.92 ± 0.01	3.98 ± 0.15 (4)	2.47 ± 0.15	2.91 ± 0.08 (3)	<0.01	<0.001
7	2.86 ± 0.23	4.15 ± 0.41 (4)	1.93 ± 0.10	2.14 ± 0.24 (4)	<0.001	<0.001
10	3.05 ± 0.05	3.92 ± 0.21 (4)	1.79 ± 0.04	1.88 ± 0.11 (4)	<0.001	<0.001
17	3.17 ± 0.31	4.21 ± 0.14 (4)	1.53 ± 0.16	1.72 ± 0.11 (4)	<0.001	<0.001
24	3.01 ± 0.12	3.91 ± 0.27 (4)	1.37 ± 0.13	1.46 ± 0.10 (4)	<0.001	<0.001
31	3.03 ± 0.25	3.85 ± 0.23 (4)	1.23 ± 0.13	1.35 ± 0.20 (3)	<0.001	<0.001
38	3.20 ± 0.23	3.89 ± 0.11 (4)	1.35 ± 0.25	1.48 ± 0.17 (3)	<0.001	<0.001

TABLE II

THE PLASMA GLUCOSE AND SERUM INSULIN CONCENTRATIONS OF STEPTOZOTOCIN-DIABETIC AND CONTROL RATS

Each result is given as the mean ± standard deviation for three or four animals.

Time following injection (days)	Plasma glucose concn. (mM)		Serum insulin concn. (munits · l^{-1})	
	Control animals	Diabetic animals	Control animals	Diabetic animals
1	6.8 ± 0.2	18.3 ± 2.3	22 ± 7	11 ± 4
3	6.7 ± 0.7	22.8 ± 3.0	32 ± 14	<5
7	7.3 ± 0.0	24.4 ± 1.2	43 ± 12	<5
10	7.4 ± 0.3	28.6 ± 4.8	24 ± 3	<5
17	7.1 ± 0.2	31.1 ± 1.7	15 ± 3	<5
24	7.0 ± 0.5	36.7 ± 4.9	27 ± 7	<5
31	7.1 ± 0.8	36.9 ± 5.7	27 ± 9	<5
38	7.3 ± 0.1	40.6 ± 7.0	35 ± 6	<5

animals, however, nearly all the transhydrogenase activity of liver appears to be available and functional as is shown by the failure to increase the enzyme activity following Polytron treatment.

When the enzyme activity is plotted in terms of unit liver protein, then essentially similar results are obtained (data not shown).

One day (24 h) after the injection of streptozotocin the mean blood glucose in the three animals killed was 18.2 mM; this compares with the blood glucose concentrations of 6.8 mM in the control animals. The blood glucose concentration of the diabetic animals then progressively increased with time (Table II). The serum insulin concentration in the diabetic animals was significantly

TABLE III

THE EFFECT OF A SINGLE INJECTION OF STREPTOZOCIN (60 mg/kg BODY WEIGHT) ON THE ACTIVITY OF GLUTATHIONE-INSULIN TRANSHYDROGENASE OF RAT KIDNEY

The kidneys were homogenized in 0.25 M sucrose/5 mM EDTA/5 mM Tris buffer, pH 7.5, with a Polytron homogenizer. Each result is given as a mean ± standard deviation for three or four animals (shown in brackets). The *P* values were determined by the use of Student's *t*-test.

Time after injection (days)	Glutathione-insulin transhydrogenase activity				
	Control animals		Diabetic animals		*P* values A—C
	A Units/mg DNA	B Units/mg protein	C Units/mg DNA	D Units/mg protein	
1	0.98 ± 0.21	24.1 ± 3.2 (4)	1.28 ± 0.13	23.5 ± 2.1 (4)	<0.05
3	1.05 ± 0.13	25.7 ± 1.8 (4)	0.82 ± 0.08	24.0 ± 0.9 (3)	<0.025
7	0.96 ± 0.08	24.5 ± 2.0 (4)	0.68 ± 0.11	21.0 ± 1.1 (4)	<0.01
10	1.02 ± 0.12	22.1 ± 4.1 (4)	0.57 ± 0.04	16.5 ± 2.1 (4)	<0.001
17	1.10 ± 0.18	20.5 ± 3.5 (4)	0.49 ± 0.08	15.0 ± 0.8 (4)	<0.001
24	0.95 ± 0.09	20.1 ± 1.8 (4)	0.43 ± 0.19	12.5 ± 1.2 (4)	<0.001
31	0.88 ± 0.10	19.5 ± 2.3 (4)	0.44 ± 0.03	12.1 ± 0.7 (3)	<0.001
38	0.90 ± 0.20	21.0 ± 0.9 (4)	0.41 ± 0.08	11.0 ± 1.3 (3)	<0.01

decreased 24 h after the injection of streptozotocin and remained at a decreased level throughout the course of the experiment (Table II). There is some variation in serum insulin levels of the control animals. This variation most likely occurred because the serum samples were obtained from animals in ad lib fed state which is a known cause of such a variation in insulin levels.

As noted above, the kidneys had been stored at −20°C for at least 24 h before the initial measurement of transhydrogenase activity. Since this freezing and thawing results in the loss of the latency of transhydrogenase [10], it was not possible to determine the relative proportion of 'functional' and latent enzyme activity in the kidneys. The total activity was therefore measured in homogenates following Polytron treatment and the results are shown in Table III. The pattern obtained resembles that of the hepatic enzyme except that the injection of streptozotocin results in an initial increase in the activity of the kidney transhydrogenase. After 48 h, however, there was a significant decrease ($P < 0.025$) in the enzyme activity as compared with the controls. The activity then decreased rapidly for about 10 days and then gradually with time until 38 days after the streptozotocin injection when the activity had fallen to 50% that of the untreated controls.

Discussion

Previous measurements of the effect of diabetes on the activity of GSH-insulin transhydrogenase in the livers of diabetic animals were made at a fixed, prolonged time after the induction of diabetes. In the present studies we have measured the effect of the development of diabetes on the activity of the enzyme. The data clearly show that a single injection of streptozotocin at a diabetogenic concentration rapidly leads to a reduction in the activity of the enzyme and that this decrease continues with time until it stabilizes at a lowered level. The question that naturally arises is whether the observed effects are due to the direct action of streptozotocin or are related to the diabetic state.

There is evidence that following the intravenous administration of ^{14}C-labeled streptozotocin, high levels of bound radioactivity are present in the liver and kidney [16,17] and it has been suggested that the liver is the major site of metabolism of streptozotocin [18]. Furthermore it is known that the administration of streptozotocin to rats rapidly leads to metabolic changes such as a decrease in red blood cell GSH [19] and an increased in the RNA content and protein/DNA ratio of the kidney [20]. However, it does appear that streptozotocin is subjected to rapid metabolic degradation by the rat [18] and although the initial decrease in transhydrogenase activity may possibly be related to the acute toxic effects of streptozotocin, it is more likely that the continuing fall in enzyme activity seen throughout the period of the experiment is related to the hyperglycemia and hypoinsulinemia of diabetes.

Diabetes apparently has little effect on the overall rate of synthesis of hepatic tissue protein in the rat [21,22] but decreases in the activity of a number of individual enzymes, in particular glucose-catabolizing enzymes [23] and triglyceride lipase [24], have been reported. The present study confirms that a decrease in hepatic GSH-insulin transhydrogenase activity occurs in diabetes.

In contrast to the liver, the kidney shows an increased rate of protein synthesis in diabetes [20] and in the present studies it was noted that the average weight of the kidneys increased despite the generally wasting conditions. In common with the hepatic enzyme, however, renal GSH-insulin tranhydrogenase activity decreased significantly in the diabetic animals. The liver and kidneys are the major organs involved in the removal of insulin from the circulation [25] and the decrease in transhydrogenase activity in both these organs in the diabetic state would agree with the hypothesis that circulating insulin has a role in the regulation of this enzyme. There are reports, however, that in addition to insulin, the thyroid hormones may control transhydrogenase activity [26,27], and studies are currently in progress to further define the control mechanisms in the insulin-degrading process.

One interesting aspect of the work reported here is the alteration in the proportion of the hepatic enzyme occurring in a latent state as shown by the treatment with the Polytron homogenizer. Whereas 25 percent of GSH-insulin transhydrogenase activity in the case of the control animals was in a latent state, in the case of the diabetic animals (having hypoinsulinemia and hyperglycermia) virtually all of the enzyme was free and functional. This is in direct contrast to the results obtained with the liver of the *ob/ob* mouse (characterized by hyperinsulinemia and hyperglycemia) where only about half of the total activity was in the free state [8]. This provides strong evidence that the circulating insulin concentrations, and not the blood glucose level (since both models show hyperglycemia), modulate the activity of transhydrogenase as well as the form in which the enzyme exists in the liver, and this might constitute another regulatory mechanism over GSH-insulin transhydrogenase activity. It is possible that during insulin-sufficiency or excess states, because of increased synthesis of the enzyme, there is a deposition of the latent form, whereas during insulin-insufficiency states, because of lack of synthesis of the enzyme, there is mobilization of the latent form to the free form in order for the cell to function which results in the disappearance of the latent form (cf. Refs. 28 and 29).

The exact cause of this latency is a matter of speculation at present but presumably it is related to the spatial organization of GSH-insulin transhydrogenase in the microsomal membrane. A clue as to a possible mechanism for the latency has come from the observation that while lysolecithin and phosphatidic acid cause inhibition of purified transhydrogenase, a number of other phospholipids cause an increase in the transhydrogenase activity of microsomal fractions [30]. Furthermore, treatment of hepatic 'microsomes' with phospholipases leads to a release of transhydrogenase activity [10]. It is therefore possible that the latency of GSH-induling transhydrogenase could be caused, at least in part, by the nature and content of pbospholipids in microsomal membranes. In fact, such differences in the phospholipid composition in microsomes of livers between obese (*ob/ob*) and lean mice have recently been found [9].

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