Insulin-dependent changes in lysosomal cathepsin D activity in rat liver, kidney, brain and heart

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Summary. Streptozotocin-induced diabetes resulted in a decrease in the cathepsin D activity (free and total) in rat liver, kidney, brain and heart with a concomitant increase in tissue protein content and amino acids pool size. Treatment with insulin brought about the restoration of the cathepsin D activity to normal or greater than normal levels; tissue protein

content and amino acids pool size also returned to normal values.

Key words: Streptozotocin, diabetes, insulin treatment, cathepsin D activity.

Hormonal influences on cellular metabolism have been well documented [1, 2]. However, there are only a few reports in the literature relating to the *in vivo* regulation of lysosomal enzyme activity by the hormonal state [3]. These pertain mainly to thyroid hormones and the pancreatic hormone, insulin. While studies dealing with thyroid hormone effects are of a more direct nature [4–9], in the case of insulin, there have been only a limited number of studies which have examined directly insulin-dependent changes in lysosomal enzyme activity [10–13]. Thus, most of the earlier studies have made use of either perfusion or organ culture techniques in normal rats to examine the influence of insulin on amino acid metabolism or autophagic vacuoles [14–18]; interestingly, most of the effects of insulin observed in these studies could be duplicated by glucagon [18]. More direct experiments have made use of genetically diabetic or experimental diabetic animals to study mostly lysosomal enzyme of carbohydrate metabolism [10, 11]. The only reports dealing with lysosomal proteolytic enzymes are by Chua et al. [12] and Stauber and Fritz [13] using alloxan and streptozotocin-diabetic animals where cathepsin D, B and H-like activities were examined in heart and skeletal muscle respectively. It is, therefore, apparent that reports dealing with insulin-status-dependent effects on lysosomal proteolytic activity are scarce. Since insulin plays an important role in tissue protein metabolism [1], it is of interest to examine the possible correlation between the insulin status of the animal and its effects on the cathepsin D activity in the tissues. This paper describes

studies on cathepsin D activity in various tissues of streptozotocin-diabetic rats and the influence of insulin treatment on the restoration of these activities.

Materials and methods

Animals

Male albino rats of Wistar strain weighing between 200–220 g were made diabetic by intraperitoneal (i.p.) injection of streptozotocin (STZ) at a dose of 65 mg/kg body weight [19]. STZ solution was made fresh prior to use in citrate buffer (50 mmol/l, pH 4.5) and used within 3–4 min. Control animals received only citrate buffer. The diabetic state was ascertained in terms of loss of body weight, polyuria, glucosuria and polydipsia. Additionally, blood glucose levels were also monitored routinely [20]. A group of diabetic animals received NPH insulin (The Boots Company Ltd Bombay, India) subcutaneously (s.c.) at a dose of 0.5–0.6 U per 100 g body weight twice daily for 7 consecutive days starting at the end of 3 weeks of STZ treatment; the last injection was given 18 h prior to killing.

Methods

Animals were killed at the end of one month after treatment with STZ or vehicle, by decapitation. Liver, kidney, brain and heart were quickly removed and homogenised in chilled (0–4°C) 250 mmol/l sucrose in a Potter-Elvehjem type glass-teflon homogeniser at 500 rpm with two up and down strokes to obtain 10% (w/v) homogenates. This procedure is adequate to rupture the cells but inflicts minimal damage to the integrity of the subcellular structures [9, 21]. The tissue homogenates were used within 15–20 min for estimation of 'free' cathepsin D activity (details given below).

Cathepsin D activity

The basal medium used for the determination of cathepsin D activity consisted of 0.5 ml of haemoglobin substrate solution and 0.5 ml of tissue homogenate. The haemoglobin (2 mg/ml) substrate solution was made up in 50 mmol/l acetate buffer, pH 3.8 containing 250 mmol/l sucrose. The final pH of buffered-substrate was adjusted to 3.8 with acetic acid which simultaneously resulted in denaturation of haemoglobin [22].

For measurement of the 'free' activity, after pre-incubation of 0.5 ml homogenates for 2 min at 37 °C, the reaction was initiated by addition of 0.5 ml of denatured buffered-substrate and the incubation continued for 10 min. The reaction was terminated by the addition of 2.0 ml of 10% (w/v) trichloroacetic acid (TCA). The sample

tubes were allowed to stand on ice for 15 min and filtrates were collected using Whatman No.1 filter paper. Aliquots of filtrates were used for determination of Folin-positive materials by the method of Spies [23].

For 'total' cathepsin D activity, the complete release of the enzyme was ensured by subjecting diluted homogenates (1:5 with 250 mmol/l sucrose) to six cycles of freeze-thaw operations. Additionally, 0.02% Triton X-100 was also included in the haemoglobin substrate. The assay of the enzyme was performed as stated above.

In separate experiments, it was ascertained that the optimum enzyme activity and linear reaction rate were obtained at 2 mg/ml of haemoglobin concentration and this concentration was hence used in all subsequent assays.

The aspartic proteinase nature of cathepsin D activity was also evaluated in separate experiments by examining the inhibitory pattern of the enzyme in the presence of pepstatin. Thus, pepstatin at a concentration of 50 µmol/l and 100 µmol/l caused 82% and 90% inhibition of cathepsin D activity, respectively, in all the tissues indicating that true cathepsin D alone is responsible for the haemoglobin splitting activity at pH 3.8.

Table 1. Experimental animal data

Parameter	Rats		
	Normal (10)	Diabetic (10)	Diabetic + Insulin (10) ^c
Body weight (g)	325.0 ± 5.43	210.0 ± 4.30 ^a	279.0 ± 4.85 ^{a, b}
Urine volume (ml/24 h)	2.42 ± 0.28	150.0 ± 9.22 ^a	5.6 ± 1.20 ^b
Urine glucose (mg/ml)	-	81.7 ± 0.36 ^a	ND
Urinary glucose (g/24 h)	-	12.2 ± 0.71 ^a	-
Blood glucose (mmol/l)	6.41 ± 0.22	27.12 ± 2.04 ^a	8.61 ± 0.49 ^{a, b}

Diabetes was induced by injecting i.p. streptozotocin (65 mg/kg body weight) to male Wistar rats. Figures in parentheses represent the number of animals used. Results are given as mean ± SEM.

^a $p < 0.001$ compared to normal; and ^b $p < 0.001$ compared to diabetic. ND: Not detected. ^c 0.5–0.6 units of NPH insulin was injected s.c. twice daily for 7 days

Amino acids pool size

For the estimation of free amino acids pool size, 0.5 ml aliquots of homogenates were deproteinised with 10% TCA and Folin-positive materials were determined from filtrates as described above [23].

Protein was measured by the method of Lowry et al. [24] employing bovine serum albumin as the standard.

Results

It can be noted (Table 1) that the diabetic rats lost considerable weight (115 g) compared to normal rats and insulin treatment was effective in restoring their body weights almost to the normal values. Diabetic rats were

Table 2. Effect of streptozotocin-induced diabetes on cathepsin D activity, protein content and free amino acids pool size in rat liver, kidney, brain and heart

Tissue	Animals ^a	Cathepsin D activity (µg Tyrosine/10 min/mg protein)		Tissue protein content ^f (mg/g tissue)	Amino acids pool ^f (µg Tyrosine/g tissue)
		Free	Total		
Liver	Normal (20)	1.22 ± 0.08	4.72 ± 0.23	143.3 ± 13.0	468.0 ± 27.8
	Diabetic (10)	0.45 ± 0.01 ^d	2.48 ± 0.26 ^d	213.1 ± 6.2 ^d	701.5 ± 26.7 ^d
	Diabetic + Insulin (16)	1.63 ± 0.25 ^e (NS ₁)	6.17 ± 0.22 ^{d, e}	147.7 ± 3.7 ^e (NS ₁)	443.6 ± 51.0 ^e (NS ₁)
Kidney	Normal (32)	2.36 ± 0.09	7.31 ± 0.33	119.6 ± 3.7	458.3 ± 20.3
	Diabetic (10)	1.21 ± 0.06 ^d	5.86 ± 0.40 ^b	132.2 ± 5.8 (NS ₁)	732.6 ± 21.8 ^d
	Diabetic + Insulin (12)	3.14 ± 0.19 ^{d, e}	8.94 ± 0.53 ^{c, e}	117.4 ± 6.1 (NS ₁ , NS ₂)	442.8 ± 28.3 ^e (NS ₁)
Brain	Normal (22)	0.98 ± 0.08	2.55 ± 0.29	93.1 ± 5.5	123.6 ± 10.8
	Diabetic (10)	0.48 ± 0.04 ^d	1.57 ± 0.13 ^b	130.7 ± 7.4 ^d	266.2 ± 8.6 ^d
	Diabetic + Insulin (9)	1.06 ± 0.07 ^e (NS ₁)	2.67 ± 0.17 ^e (NS ₁)	85.2 ± 2.0 ^e (NS ₁)	115.0 ± 5.9 ^e (NS ₁)
Heart	Normal (30)	0.60 ± 0.04	1.56 ± 0.10	90.6 ± 4.6	116.7 ± 6.1
	Diabetic (9)	0.49 ± 0.09 (NS ₁)	0.96 ± 0.11 ^d	96.3 ± 6.9 (NS ₁)	207.5 ± 13.7 ^d
	Diabetic + Insulin (10)	1.60 ± 0.06 ^{d, e}	2.92 ± 0.20 ^{d, e}	82.8 ± 6.8 (NS ₁ , NS ₂)	101.5 ± 4.3 ^e (NS ₁)

^a Figures in parentheses represent the number of animals. The values are given as mean ± SEM. ^b $p < 0.01$, ^c $p < 0.002$ and ^d $p < 0.001$ compared with corresponding normal values respectively. ^e $p < 0.001$ compared with corresponding diabetic rats. ^f Number of observations for these studies was 5–10.

NS₁: Not significant compared with corresponding normal rats and NS₂: Not significant compared with corresponding diabetic rats

also characterised by polyuria, glucosuria and polydipsia. Thus the daily urine volume was about 150 ml for diabetic animals and urinary glucose level was approximately 80 mg/ml, resulting in urinary excretion of 12 g of glucose per day. Additionally, the diabetic animals displayed a 4-fold increase in blood glucose levels. These observations are in conformity with reports of other investigators [19, 25, 26] and correlate well with the established parameters of animal diabetes models [26]. Treatment with insulin effectively cured practically all the symptoms including blood glucose levels, which were only slightly higher than those of the normal control rats (Table 1).

The data on the cathepsin D activity, tissue protein content and amino acids pool size in the four tissues under investigation from normal, diabetic and insulin-treated diabetic animals are summarised in Table 2. It is evident that the 'free' cathepsin D activities decreased in all four tissues. The extent of decrease varied from 18–63%. The decline in 'free' enzyme activity was the highest in liver and the least in heart with a uniform 50% decrease in kidney and brain. The 'total' cathepsin D activity was also decreased on an average by 40–48% in liver, brain and heart while decrease in the kidney was found to be of a lesser magnitude (20% decrease). Treatment with insulin restored these activities to levels comparable to normal in the case of brain tissue, whereas in other tissues these values were elevated beyond normal levels (30–165% increase). Interestingly, concomitant with a decrease in cathepsin D activities in the diabetic animals there was a tendency towards increased protein content in the tissues from 5–50%. The free amino acids pool size was found to be increased from 50% to 115% (Table 2). Elevated tissue protein content and amino acids pool size values were restored to the normal levels following insulin treatment.

Discussion

The present studies were undertaken to examine the insulin-dependent changes in lysosomal cathepsin D activity in various tissues. The results have clearly shown that 'free' as well as 'total' cathepsin D activities were significantly decreased in all four tissues of one-month STZ-diabetic animals. Insulin treatment restored these activities to normal levels in the case of brain and to levels greater than normal in the case of liver, kidney and heart. Earlier, Skoza et al. [11] have reported the decrease in lysosomal enzymes of carbohydrate metabolism in the hearts of genetically diabetic mice. Lowering of lysosomal enzymes of carbohydrate metabolism in kidneys from diabetic rats [27] was also reported. Similar to our findings, decreased cathepsin D levels in hearts from alloxan diabetic rats was reported by Chua et al. in their short-term studies; the effect was accentuated following perfusion [12].

More recently, Stauber and Fritz [13] have also reported decreased cathepsin B and H-like activities in skeletal muscle of STZ-diabetic rats.

A decrease in the cathepsin D activities in the tissues of diabetic animals was accompanied by a concomitant increase in the amino acids pool size in general and a tendency towards increased tissue protein content (Table 2). Treatment of the diabetic animals with insulin restored the amino acids pool size and tissue protein levels to normal values (Table 2). Increased liver protein content with decreased protein turnover rates in liver and kidney from STZ-diabetic animals have been reported by Hayase et al. [28]. Similarly, alloxan-diabetic rats have also been reported to show increased liver protein content [25]. A rise in tissue proteins in diabetic animals could perhaps be ascribed to the decreased proteolysis. The overall decline in both 'free' and 'total' activities of cathepsin D observed in the present studies (Table 2) suggest such a possibility. The increase in amino acids pool size, on the other hand, could possibly be related to their non-utilisation for protein synthesis. In this connection, it is of interest to note that insulin is known to alter the circulating levels of amino acids and their tissue uptake [18, 29].

The bound activity ($\frac{\text{'total'-'free'}}{\text{'total'}} \times 100$) of lysosomal

enzymes is generally taken as an index of intactness of the granules [3, 9, 30]. Although not given in the table (Table 2), this ratio can be easily determined from data therein. Thus, for control and insulin treated animals, these ratios were in close agreement for practically all the tissues; in diabetic animals, the ratios were generally even higher. It is, therefore, clear that in no experimental group had the lysosomes suffered a structural damage.

In separate experiments we have verified that the optimum haemoglobin substrate concentration remains unaltered for cathepsin D activity from diabetic animals (results not given) indicating that the kinetic properties of the enzyme were not altered.

The results of the present studies have thus shown a direct relationship between the tissue protein metabolism and cathepsin D activity as influenced by the insulin status of the animals.

The observed decrease in lysosomal cathepsin D levels in all the tissues examined from STZ-diabetic animals and its restoration by insulin treatment is not surprising since the presence of insulin receptors in all these tissues has been reported [31, 32].

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