Influence of Streptozotocin- and Alloxan-Induced Diabetes in the Rat on Collagenase and Certain Lysosomal Enzymes in Relation to the Degradation of Connective Tissue Proteins

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Summary. The activity of collagenase and certain lysosomal hydrolases (cathepsin B₁, cathepsin D, β -glucuronidase and β -N-acetyl glucosaminidase) was studied in serum and tissues of rats with streptozotocin- or alloxan-induced diabetes. The activity of serum lysosomal enzymes was increased in both groups ($p < 0.05$). Both streptozotocin- and alloxan-diabetic animals showed significantly higher dermal collagenase activity than those of controls ($p < 0.01$), but the liver and spleen showed similar activities; there was a significant decrease in the renal collagenase activity of streptozotocin-diabetic rats ($p < 0.05$). Comparison of the alloxan- or streptozotocin-treated groups with control animals showed an increase in lysosomal enzymes (cathepsin B₁, cathepsin D, β -glucuronidase

and β -N-acetyl glucosaminidase in skin, liver and spleen) ($p < 0.05$) but β -N-acetyl glucosaminidase was unchanged in the spleen of streptozotocin-diabetic rats. There was no difference in renal cathepsin B₁ and D in control versus alloxan-diabetic rats, but there was an increase in β -glucuronidase and β -N-acetyl glucosaminidase ($p < 0.05$). The streptozotocin-diabetic animals showed decreased activities of renal lysosomal enzymes ($p < 0.05$), but similar activity of cathepsin D to the control animals.

Key words: Diabetes, rat, collagenase, cathepsin B₁, cathepsin D, β -glucuronidase, β -N-acetyl glucosaminidase, lysosomal hydrolases, connective tissue, streptozotocin, alloxan.

Activities of the plasma or serum lysosomal enzymes such as β -glucuronidase and β -N-acetyl glucosaminidase have been shown to be elevated in diabetic patients [4, 23] and in various animal models including chemically induced diabetes in rats [35] and Chinese hamsters [7]. The activity of collagenolytic enzyme capable of solubilising collagen is also increased in the gingiva of animals rendered diabetic by alloxan [29]. Changes in the activities of lysosomal hydrolases involved in the degradation of connective tissue proteins such as, proteoglycans [20], glycoproteins [21] and collagen [8] may play important roles in the pathophysiology of the diabetic sequelae. Studies have therefore been initiated on the levels of collagenase and of other lysosomal enzymes (cathepsin B₁, cathepsin D, β -glucuronidase and β -N-acetyl glucosaminidase) in serum and tissues of animals with two types of experimentally induced diabetes.

Materials and Methods

Four-month-old male albino rats were divided into three groups: control animals, streptozotocin-treated and alloxan-treated. Rats were starved for 24 h and given an IP injection of alloxan in 0.154 mol/l

NaCl (20 mg/100 g body weight) or an IV injection of streptozotocin (Upjohn, Kalamazoo, Michigan, USA) in isotonic saline containing 1 mol/l citric acid (pH 4.5) (6.5 mg/100 g body weight) [24]. All animals received commercial rat feed (Hindustan Lever, Bombay) and water ad libitum. Body weights were recorded at weekly intervals. Blood samples were taken weekly from the tails of fasted rats for measurement of glucose [24]. At the end of the experimental period (3 weeks), 12 rats in each group were killed by decapitation and the skin, liver, kidney and spleen were removed immediately in ice-cold containers and stored at -20°C . Blood was collected by heart puncture before killing the rats and allowed to clot after standing at room temperature for 2–4 h. The serum was separated by centrifugation at low speed and stored at -20°C until assayed. For the convenience of analysis, tissues or blood from two rats were pooled together to make one sample thus making six samples in each group. Tissue samples were analysed for collagenase, cathepsin B₁, cathepsin D, β -glucuronidase and β -N-acetyl glucosaminidase activities and serum was analysed for these enzymes except collagenase.

Preparation of Enzyme Extract from Tissues

The skins were shaved and sliced approximately 5–10 μ with a microtome at 0°C . The enzyme extract was prepared by homogenising the weighed tissue samples separately in a Potter Elvehjem type homogenizer (Remi, Bombay, India) using ice-cold 0.25 mol/l sucrose solution. Conditions of homogenization were standardised for speed and time of homogenization and the volumes of medium used were identical in all cases. The homogenates were then centrifuged for 10 min at 600 g. The residue containing nuclei, unbroken cells and plasma

Table 1. Effect of streptozotocin- and alloxan-induced diabetes on the serum activities of cathepsin B₁, cathepsin D, β -D-glucuronidase and β -N-acetyl glucosaminidase

Enzyme	Control rats	Streptozotocin	Alloxan
Cathepsin B ₁ ($\mu\text{m p-nitroaniline} \cdot \text{h}^{-1} \cdot 100 \text{ mg protein}^{-1}$)	15.4 $\pm$ 0.5	17.4 $\pm$ 0.6 ^a	18.6 $\pm$ 0.6 ^a
Cathepsin D ($\mu\text{m tyrosine} \cdot \text{h}^{-1} \cdot 100 \text{ mg protein}^{-1}$)	10.4 $\pm$ 0.3	12.1 $\pm$ 0.4 ^b	11.8 $\pm$ 0.4 ^a
β -glucuronidase ($\mu\text{m p-nitrophenol} \cdot \text{h}^{-1} \cdot 100 \text{ mg protein}^{-1}$)	2.6 $\pm$ 0.1	3.2 $\pm$ 0.1 ^b	3.1 $\pm$ 0.1 ^b
β -N-acetyl glucosaminidase ($\mu\text{m p-nitrophenol} \cdot \text{h}^{-1} \cdot 100 \text{ mg protein}^{-1}$)	2.8 $\pm$ 0.1	3.6 $\pm$ 0.1 ^b	3.5 $\pm$ 0.1 ^b

Results expressed as mean $\pm$ SEM; six samples with two animals sera in each sample ^a $p < 0.05$; ^b $p < 0.01$

membrane were discarded and the supernatant was again centrifuged at 16,000 g for 30 min at 4°C. The supernatants were used as the source of free activities of lysosomal hydrolases [28] and enzyme activities were estimated in presence of 0.2% (w/v) Triton X 100.

For collagenase, the weighed tissue samples were homogenised separately in a Teflon glass homogeniser using ice-cold 0.5 mol/l Tris-HCl buffer (pH 7.8) containing NaCl (0.2 mol/l) and CaCl₂ (5 mmol/l) and the extract was frozen and thawed three times and centrifuged at 16,000 g for 30 min. The supernatant containing 0.5–1.5 mg protein/ml was used for collagenase assay.

Preparation of Substrate for Collagenase

Twenty-five-day-old guinea pigs, weighing 225–250 g, were injected IP with 200 μCi of ¹⁴C glycine (Bhabha Atomic Research Centre, Bombay) in sterile 0.154 mol/l NaCl. Six hours later the animals were anaesthetized with ether and killed by decapitation. The skins were shaved, minced with NaCl (0.5 mol/l) in the cold and the neutral salt soluble collagen was extracted and purified as described previously [25]. The collagen preparations were freeze-dried and stored at –20°C until use. The specific radioactivity was found to be in the range of 2500 cpm/mg of collagen and the purity of the collagen was confirmed by disc electrophoresis in 5% polyacrylamide gels containing sodium dodecyl sulphate [25].

Collagenase Assay

The activity of collagenase was assayed as described by Terato et al. [34]. For enzyme assay, 0.1 ml of 0.4% (w/v) labelled collagen dissolved in acetic acid (5 mmol/l), 0.1 ml of 0.1 mol/l Tris-HCl buffer (pH 7.8) containing NaCl (0.4 mol/l) CaCl₂ (10 mmol/l) and glucose (1 mol/l) and 0.2 ml of enzyme solution to be tested were mixed and incubated for 4 h at 35°C (a temperature between the denaturation temperature of the native collagen and its enzyme digests). The enzymic reaction was stopped by adding 20 μl of o-phenanthroline (80 mmol/l) dissolved in 50% dioxane and the reaction products were extracted by vigorous shaking after the addition of 0.4 ml of dioxane and centrifuged at 6000 rev/min for 20 min to precipitate the residual undigested collagen. The supernatant (0.5 ml) was added to 10 ml of Brays solution (Nuclear Enterprises, Edinburgh, Scotland, UK) and assayed for ¹⁴C radioactivity in an automatic liquid scintillation system LSS 34 (Electronics Corporation of India, Hyderabad). To check the interference by denatured collagen, control assays containing 25 μg of trypsin (2 $\times$ crystalline) were performed with each series of

assays. After subtracting water and trypsin blank, the results were expressed as cpm/mg protein. To verify that the enzyme assayed was a true collagenase, collagen and its degradation products were examined by disc gel electrophoresis.

Cathepsin B₁ activity was measured by the method of Barret [3] using N- α -benzoyl-DL-arginine p-nitroanilide HCl (BAPA) as substrate. Cathepsin D assay was carried out using 1.5% haemoglobin as substrate and estimating tyrosine liberated by the method of Sapolsky et al. [31]. β -glucuronidase activity was measured by the method of Kawai and Anno using p-nitrophenyl β -D-glucuronide as the substrate [18]. β -N-acetyl glucosaminidase was assayed as described by Rosenbilt et al. [30] using p-nitrophenyl N-acetyl β -D-glucosaminide as substrate. Protein content was determined by the method of Lowry et al. [19] using bovine serum albumin as a standard.

Statistical Methods

The results evaluated using the Student's t-test are expressed as mean $\pm$ SEM. The differences were regarded as significant at the level $p < 0.05$.

Results

Streptozotocin- and alloxan-treated animals lost weight during the experimental period. The fasting blood glucose was raised in all experimental rats (control: 5.3 ± 0.4 ; streptozotocin-diabetic: 22.1 ± 0.9 and alloxan-diabetic: 16.4 ± 0.8 mmol/l respectively).

The activities of serum cathepsin B₁, cathepsin D, β -glucuronidase and β -N-acetyl β -glucosaminidase were increased significantly in both streptozotocin- and alloxan-induced diabetic animals compared with controls (Table 1).

The activity of free collagenase increased significantly in the skins of both diabetic groups. However the activity was reduced in the kidney of streptozotocin-diabetic rats while in the liver and spleen no significant changes of collagenase were observed. The activity was not altered in liver, kidney and spleen of the alloxan-diabetic group. The mode of cleavage of collagen by the collagenase from tissue homogenates of diabetic rats was characterized by disc electrophoresis on sodium dodecyl sulphate polyacrylamide gels. The gel electrophoretic patterns indicated well characterized TC_A and TC_B fragments (three-fourth and one-fourth of the intact collagen molecule respectively) supporting that the enzyme assayed was collagenase.

Significant increase in the activity of cathepsin B₁ was observed in skin, liver and spleen of both types of diabetes. The kidneys of streptozotocin-diabetic rats showed lower enzyme activity whereas alloxan diabetic animals had similar activity as those in the non-diabetic animals (Table 2).

Diabetes rendered by streptozotocin and alloxan caused significant elevation in the cathepsin D activity in the skin, liver and spleen. No difference in renal cathepsin D activity was detected between control and alloxan- or streptozotocin-induced diabetic rats (Table 2).

The levels of β -glucuronidase in both type of experimentally induced diabetes exceeded those in controls in

Table 2. Effect of streptozotocin- and alloxan-induced diabetes on the activities of collagenase, cathepsin B₁, cathepsin D, β -glucuronidase and β -N-acetyl glucosaminidase in skin, liver, kidney and spleen

Enzyme	Group	Skin	Liver	Kidney	Spleen
Collagenase (cpm/mg protein)	Control	566 $\pm$ 12	121 $\pm$ 5	147 $\pm$ 5	138 $\pm$ 6
	Streptozotocin	738 $\pm$ 15 ^b	138 $\pm$ 8	104 $\pm$ 4 ^b	149 $\pm$ 5
	Alloxan	694 $\pm$ 14 ^b	130 $\pm$ 6	136 $\pm$ 4	156 $\pm$ 8
Cathepsin B ₁ (μ m p-nitroaniline h ⁻¹ · 100 mg protein ⁻¹)	Control	51.9 $\pm$ 2.8	21.5 $\pm$ 1.7	32.4 $\pm$ 2.2	40.4 $\pm$ 2.7
	Streptozotocin	65.8 $\pm$ 5.3 ^a	28.7 $\pm$ 1.4 ^a	26.3 $\pm$ 1.2 ^a	52.1 $\pm$ 3.4 ^a
	Alloxan	61.2 $\pm$ 3.1 ^a	29.0 $\pm$ 3.1 ^a	30.3 $\pm$ 3.2	56.5 $\pm$ 4.1 ^a
Cathepsin D (μ m tyrosine · h ⁻¹ · 100 mg protein ⁻¹)	Control	36.1 $\pm$ 3.2	24.3 $\pm$ 1.1	35.6 $\pm$ 2.0	28.6 $\pm$ 1.4
	Streptozotocin	50.6 $\pm$ 4.8 ^a	32.9 $\pm$ 1.7 ^b	31.1 $\pm$ 2.4	35.5 $\pm$ 2.5 ^a
	Alloxan	44.2 $\pm$ 2.1 ^a	31.4 $\pm$ 2.1 ^a	38.8 $\pm$ 2.6	39.6 $\pm$ 2.7 ^b
β -glucuronidase (μ m p-nitrophenol · h ⁻¹ · 100 mg protein ⁻¹)	Control	25.1 $\pm$ 1.7	37.6 $\pm$ 2.2	26.2 $\pm$ 1.1	62.6 $\pm$ 3.6
	Streptozotocin	31.5 $\pm$ 2.0 ^a	46.1 $\pm$ 2.8 ^a	20.3 $\pm$ 1.2 ^b	78.9 $\pm$ 5.4 ^a
	Alloxan	36.4 $\pm$ 2.4 ^b	44.4 $\pm$ 2.2 ^a	30.6 $\pm$ 1.3 ^a	80.4 $\pm$ 3.2 ^b
β -N-acetyl glucosaminidase (μ m p-nitrophenol · h ⁻¹ · 100 mg protein ⁻¹)	Control	52.1 $\pm$ 3.7	31.7 $\pm$ 2.5	162.5 $\pm$ 7.1	43.8 $\pm$ 3.2
	Streptozotocin	68.4 $\pm$ 2.9 ^b	41.2 $\pm$ 2.3 ^a	118.3 $\pm$ 6.2 ^b	37.5 $\pm$ 2.6
	Alloxan	64.5 $\pm$ 4.5 ^a	43.0 $\pm$ 2.1 ^b	183.7 $\pm$ 6.7 ^a	54.2 $\pm$ 2.7 ^a

Results expressed as mean $\pm$ SEM; six samples with two animal tissues in each sample ^a $p < 0.05$; ^b $p < 0.01$.

all the tissues with the exception of kidney of streptozotocin-diabetic rats which showed a decrease (Table 2).

Administration of streptozotocin caused a decrease in the activity of β -N-acetyl glucosaminidase in the kidney, an increase in the skin and liver but no alteration in the spleen. The activity of this enzyme increased significantly in all the tissues of alloxan-diabetic rats (Table 2).

Discussion

Diabetes-related changes in the activities of plasma or serum β -glucuronidase and β -N-acetyl glucosaminidase involved in the degradation of glycosaminoglycans and glycoproteins have been reported in diabetic patients [4, 23] and in experimentally-induced [14, 35] or spontaneously-occurring diabetes [7]. However, no information is available on the changes in collagenase and collagenolytic enzymes, namely cathepsin B₁ and D involved in collagen degradation in diabetic subjects. The present study has shown an elevation of serum lysosomal enzymes (cathepsin B₁, cathepsin D, β -glucuronidase and β -N-acetyl glucosaminidase) in both streptozotocin- and alloxan-treated rats. The increase in the serum lysosomal enzyme activities in diabetes is thought to be caused by release of these enzymes into serum from tissue lysosomes. The liver perfusion experiments reported by Tulsiani et al. [35] suggest that the liver is contributing to the elevated lysosomal hydrolase levels observed in streptozotocin-treated rats. However Chang [6] and Tarui et al. [33] believe that the increased activities of lysosomal enzymes observed in diabetic rats was a result of a decrease in their renal activities. Better control of diabetes with insulin was associated with normalization of the enzyme activities of the serum in the diabetic state suggesting that a chronic deficiency of in-

sulin may be related to increased serum lysosomal hydrolase activities. However, streptozotocin-induced and genetically diabetic *db/db* mice [35] showed lower instead of higher activity of plasma lysosomal hydrolases than the non-diabetic mice. The cause for this discrepancy could arise from difference in species or between chemically-induced and hereditary diabetes. Genetic factors also appeared to dictate the diabetes related changes in plasma lysosomal hydrolase activities. From mixing studies between normal and diabetic sera or tissue homogenates no evidence was obtained for the presence of inhibitors or activators of β -hexosaminidase in diabetic rats [1, 38].

The present investigation shows an active collagenolytic enzyme in vivo in the tissue homogenates of normal and diabetic rats. Golub et al. [15] observed that both alloxan- and streptozotocin-induced diabetes stimulated the collagenolytic activity in gingiva in tissue culture. In addition, the diabetic rats exhibited increased urinary excretion of hydroxyproline [24]. The pathophysiological role of the inhibition of collagenolytic systems in the kidney of streptozotocin-diabetic rats is unknown.

The rapid breakdown of collagen in diabetes may not result directly from collagenase activity but may be caused by other collagenolytic enzymes [5, 10, 27]. The initial breakdown of the proteoglycans by these lysosomal enzymes may open up to collagen fibres for attack by collagenase [37]. These lysosomal enzymes may also contribute to the further degradation of collagen fragments produced by the action of collagenase. The destruction of cartilage in experimental diabetes is reported due to the enzymatic degradation of proteoglycans by lysosomal enzymes [32]. Cathepsin B₁ [12] has been found to depolymerize collagen fibres [11] and it can further digest collagen to small fragments [5]. In ad-

dition cathepsin B₁ [26] and cathepsin D [2] have also been shown to digest the protein moiety of proteoglycans [2] while β -glucuronidase can digest the polysaccharide moiety suggesting that the connective tissue proteins could be digested by lysosomal hydrolases.

Diabetes induced by alloxan and streptozotocin show some differences. While the activity of lysosomal hydrolases increased or unaltered in the kidney of alloxan-diabetic rats, these enzyme activities have been decreased in streptozotocin-induced diabetes. Comparisons of different metabolic alterations produced by these two diabetogenic agents that might illuminate this question are few in number and their results are conflicting [17, 22, 36].

Decreased lysosomal enzyme activity in renal tissue of streptozotocin-diabetic rats [14] mice [13] and Chinese hamsters with spontaneous diabetes [6] shows that lysosomal enzyme deficiency could be involved in the genesis of renal microangiopathy. Depression of renal β -glucuronidase [14] and α -mannosidase was observed in streptozotocin-diabetic rats and mice [35] but not in hereditary diabetic *db/db* mice [35]. These conflicting results suggest that diabetic related changes in renal lysosomal hydrolase levels are not regulated by hyperglycaemia alone.

Although renal lysosomal enzymes are decreased in experimental diabetes there is evidence that synthetic enzymes such as lysyl hydroxylase [9] and uridine diphosphate glucose-galactosyl-hydroxylysine transferase [16] exhibit increased activities in diabetic kidneys. Differences in the effects of diabetes on these two classes of enzymes could contribute to the accumulation of connective tissue proteins which in turn have been implicated in the development of diabetic microangiopathy. The changes in these lysosomal enzymes may also reflect biochemical adjustments to the increased deposition of macromolecules in tissues.

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