Glomerular basement membrane synthesis and serum concentration of type IV collagen in streptozotocin-diabetic rats

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Summary. Glomerular basement membrane synthesis was measured in vivo in diabetic rats at various times after streptozotocin injection. Simultaneously the type IV collagen concentration in serum was determined by a radioimmunoassay specific for the terminal cross-linking domain, 7S collagen. Basement membrane collagen synthesis and the 7S collagen levels were markedly raised in diabetic animals 18–34 days after streptozotocin injection and showed a significant correlation. The results suggest that the amount of serum 7S collagen reflects increased basement membrane synthesis in a diabetic situation. Both basement membrane collagen synthesis and serum 7S collagen concentration also show a positive correlation with the blood glucose levels of diabetic rats. This indicates that the degree of metabolic dysregulation influences basement membrane synthesis.

Key words: Basement membrane synthesis, type IV collagen, 7S collagen, diabetes, streptozotocin.

Diabetes mellitus is a disease in which the structure and function of the capillary basement membrane is substantially changed. This membrane is composed of collagenous and non-collagenous proteins. The major collagen type IV is assembled in a regular network with individual molecules linked together at one terminal region by a disulphide rich domain, 7S collagen [19]. 7S collagen can be solubilized from basement membranes by proteolysis and occurs in almost all basement membranes [13]. It is a potent immunogen and cross-reacting material has been detected in serum and other biological fluids by radioimmunoassay [14]. An increased 7S collagen level was demonstrated recently in the serum of diabetic rats, which was well controlled after treatment with insulin [15]. The reason for the elevated concentration of this basement membrane component is still not clear. In the present study, we have compared the serum concentration of 7S collagen with the activity of basement membrane synthesis in renal glomeruli of diabetic rats at various times after injection of streptozotocin.

Materials and methods

Male Wistar rats (aged 6–8 weeks, 160–180 g) were rendered diabetic by IP injection of streptozotocin (75 mg/kg body weight). These rats were selected at random from a group of the same age and sex. The remaining animals were kept as non-diabetic controls. All animals received chow pellets and water ad libitum.

Four, 18 or 34 days after diabetes induction, blood glucose determinations were performed in diabetic and control rats one day before injection of the radioisotope by using a glucose oxidase kit (Boehringer, Mannheim, FRG). Animals with similar glucose levels and body weight were pooled ($n = 4$–5 rats) and were injected with a single IP dose of L-(2,3-³H)-proline (New England Nuclear, Boston, Massachusetts, USA). Diabetic and non-diabetic animals received 150 μCi/100 g body weight of the tracer. Forty-two hours after injection of the radioisotope, the animals were killed and the kidneys were removed immediately and placed on ice. Blood was collected from each rat. After clotting at room temperature, sera were stored at $-25\,°C$.

Isolation and analyses of basement membrane

Glomeruli were isolated using a sieve technique described previously [5] and basement membranes were then purified according to the detergent method of Meezan et al. [8]. Isolated basement membranes were hydrolysed with 6 mol/l HCl for 24 h at 110 °C. An aliquot of the hydrolysate (containing 0.04–0.06 mg basement membrane) was placed on an amino acid analyser (LKB 4400, LKB Biochrom, Cambridge, UK) for determination of amino acids. ³H-proline and ³H-hydroxy-proline were measured in another aliquot of the hydrolysate which was passed over a column of Dowex 50-X12 (Serva, Heidelberg, FRG) using 1 mol/l HCl as eluant as described by Brownlee and Spiro [2]. Radioactivity was determined in a liquid scintillation counter (Betaszint BF 5000, Berthold, Wildbad, FRG). Phosphorous content of isolated basement membranes was measured according to Bartlett [1].

Table 1. Body weight, blood glucose, serum creatinine and proline concentrations in the diabetic and non-diabetic rats studied

Rats	Age (weeks)	Duration of diabetes (days)	Number of experiments/rats	Body weight (g)	Blood glucose (mmol/l)	Creatinine (μmol/l)	Proline (μmol/l)
Diabetic	7	4	6/24	162 ± 31	14.65 ± 1.44	35.36 ± 5.30	166.93 ± 29.87
Non-diabetic	7		6/24	172 ± 26	4.99 ± 0.39	35.36 ± 4.42	177.52 ± 38.53
Diabetic	11	18	3/15	173 ± 18	17.15 ± 2.61	44.20 ± 6.18	172.24 ± 16.85
Diabetic	11	34	3/15	177 ± 16	17.87 ± 2.10	53.04 ± 6.18	181.07 ± 26.95
Non-diabetic	11		6/24	238 ± 23	5.32 ± 0.28	35.36 ± 5.30	163.27 ± 20.54

Results are expressed as mean ± SD; each experiment containing four to five rats

Table 2. Amino acid composition and phosphorus content of isolated basement membranes of rats at various ages and duration of diabetes

		Diabetic rats		Non-diabetic rats	
		Aged 7 weeks: diabetic for 4 days	Aged 11 weeks: diabetic for 18–34 days	Aged 7 weeks	Aged 11 weeks
Amino acids (residues/1000 amino acid residues)	Hydroxyproline	80.3 ± 4.9	82.4 ± 6.2	78.7 ± 6.3	79.2 ± 7.1
	Proline	84.4 ± 8.1	85.3 ± 7.2	83.6 ± 7.1	85.4 ± 6.9
	Glycine	192.1 ± 9.7	200.2 ± 12.4	195.3 ± 11.3	193.0 ± 10.8
	Hydroxylysine	24.9 ± 3.2	24.2 ± 4.1	23.1 ± 5.1	25.0 ± 3.9
	Lysine	28.8 ± 4.3	26.9 ± 5.2	26.8 ± 4.7	27.9 ± 4.5
Phosphorus content (μg/100 mg basement membrane)		40 ± 6	38 ± 6	38 ± 5	41 ± 6

Results are expressed as mean ± SD of six experimental and control groups (each containing four to five rats)
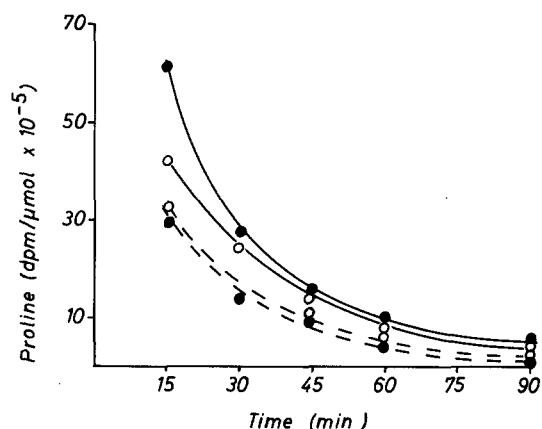
Fig. 1. Specific activity of free proline in sera of normal (——) and diabetic rats (– – – –) at various times after injection of ^{3}H-proline (150 μCi/100 g body weight). Data were obtained in 7 week- (O) and 11 week- (●) old normal rats and with diabetic rats of the same age 4 days (O) or 18–34 days (●) after streptozotocin injection

Measurement of specific activity of proline in serum

Determination of specific activity of proline in the serum of diabetic and non-diabetic rats was performed according to the method of Brownlee and Spiro [2] with minor modifications. After injection of ^{3}H-proline (150 μCi/100 g body weight), blood was taken from the tail vein at various time intervals up to 90 min. The animals were then killed by aortic puncture. Blood collected at this time was taken for the determination of the proline content of the serum. All sera were deproteinized with 5% sulphosalicylic acid. Proline radioactivity in

the deproteinized sera was measured by thin layer chromatography on silica gel sheets (Schleicher and Schüll, Kassel, FRG) in a solvent containing t-butylalcohol, ethylmethylketon, water and ammonium hydroxide (80 : 60 : 50 : 8, v/v). Total proline content of the sera was determined by an amino acid analyser (LKB 4400, LKB Biochrom, Cambridge, UK). Zero time values were extrapolated from a semi-logarithmic plot of the specific activities.

Determination of 7S collagen and statistical methods

Type IV collagen concentration in the serum was measured by a radioimmunoassay specific for 7S collagen [14]. This assay shows complete cross-reacting with intact type IV collagen [19] and with the precursor from type IV procollagen [10]. The Wilcoxon test was used for statistical evaluation.

Results

The streptozotocin-injected rats were markedly hyperglycaemic, but had only slightly increased in weight compared with control rats. Concentrations of serum proline and creatinine were similar in the diabetic and non-diabetic groups (Table 1). After IP injection of ^{3}H-proline, the serum radioactivity declined rapidly in diabetic and non-diabetic rats, and after 90 min amounted to only 3%–5% of the zero time value. The specific activity of serum proline was distinctly lower in diabetic than non-diabetic rats (Fig. 1). This difference was therefore accounted for by calculation of the radioactivity incorporated into the basement membrane as de-

Table 3. Specific activities of proline and hydroxyproline in the isolated basement membranes and serum concentrations of 7S collagen at various ages and duration of diabetes in diabetic and non-diabetic rats

Rats	Age (weeks)	Duration of diabetes (days)	Number of experi- ments/rats	Proline (dpm/μmol)	Hydroxyproline (dpm/μmol)	7S Collagen (ng/ml)
Diabetic	7	4	6/24	12582±3215	9796±2712	75.3± 7.7
Non-diabetic	7		6/24	11053± 698	9341± 639	74.5±23.3
Diabetic	11	18	3/15	7494±2225	5794±1276	42.5± 7.2
Diabetic	11	34	3/15	8207±1593	5650±1063	44.0±13.2
Non-diabetic	11		6/24	5709± 923	3710± 696	24.9± 4.9

Results are expressed as mean ± SD; each experiment containing four to five rats. dpm = desintegrations/min
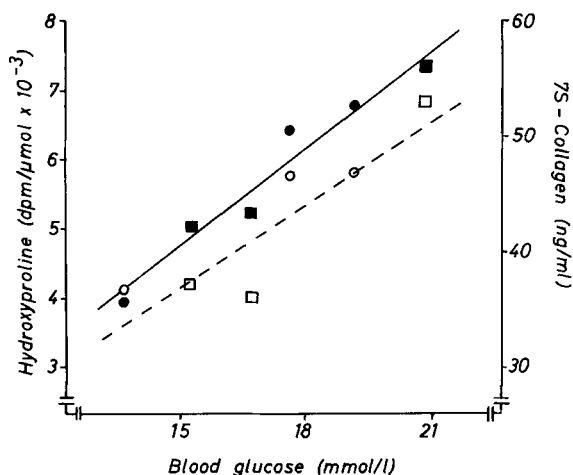
Fig. 2. Correlation between collagen synthesis in renal basement membranes (●, ■) or serum concentration of 7S collagen (O, □) and blood glucose levels in diabetic rats. Collagen synthesis was measured by determining specific activity of hydroxyproline. Blood glucose concentration was determined 1 day before injection of radioisotope. Each point represents the results of a pooled group of five animals from experiments after 18 (●, O) or 34 (■, □) days of diabetes
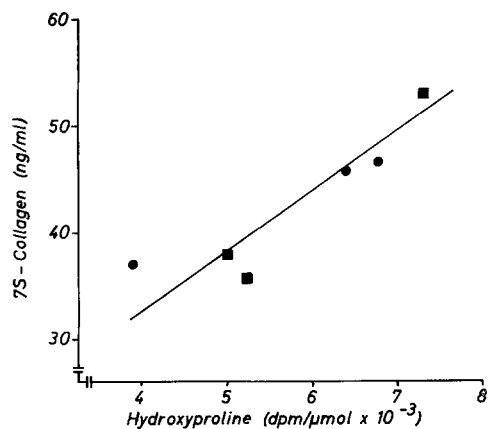
Fig. 3. Correlation between serum concentration of 7S collagen and collagen synthesis in renal basement membranes in diabetic rats 18 (●) or 34 (■) days after streptozotocin injection. Collagen synthesis was measured by determining specific activity of hydroxyproline. Each point represents the result of a pooled group of five animals from the experiments after 18 (●, O) or 34 (■, □) days of diabetes

scribed by Brownlee and Spiro [2]. The ratio of the areas under the curve of specific activity for normal and diabetic rats was calculated and found to be 1.38 in younger (duration of diabetes 4 days) and 1.79 in older rats (duration of diabetes 18–34 days). The proline and hydroxyproline activities measured in the diabetic basement membranes were corrected by the corresponding factor.

The basement membranes isolated from glomeruli of rats of varying age and duration of diabetes were equally pure as judged by their phosphorous content (Table 2), which is a sensitive parameter of contamination by cellular material [20]. The concentration of amino acid characteristics for collagenous subunits of the isolated basement membrane did not differ in the diabetic and non-diabetic groups (Table 2). The composition of the remaining amino acids did not show any difference either in these groups (data not shown). However, formation of ^{3}H-hydroxyproline was elevated by 54% in the diabetic rats 18 and 34 days after streptozotocin injection, when compared with controls ($p <$ 0.02; Table 3). Incorporation of ^{3}H-proline was only slightly raised 18 and 34 days after induction of diabetes (37%, $p < 0.05$). The concentration of 7S collagen in serum was also 74% higher in both diabetic groups than in the controls ($p < 0.01$, Table 3).

All these relative changes in basement membrane synthesis and 7S collagen were not observed in rats made diabetic for only 4 days (Table 3). Since in this experiment both the experimental and control animals were younger by 4 weeks, both groups showed higher synthetic activity and serum 7S collagen levels when compared with experiments in older rats [14].

A close correlation was observed between the specific hydroxyproline activity of basement membrane and the level of blood glucose in the diabetic rats 18 and 34 days after streptozotocin injection ($r = 0.98$; $p < 0.01$; Fig. 2). A similar correlation was found between serum 7S collagen and glucose concentration ($r = 0.88$; $p < 0.01$; Fig. 2) or formation of ^{3}H-hydroxyproline ($r = 0.91$; $p < 0.01$; Fig. 3). No significant correlation could be found between proline incorporation into the basement membrane and serum 7S collagen concentration or blood glucose.

Discussion

7S collagen is a characteristic domain of basement membrane (type IV) collagen and part of it is released into the circulation during basement membrane metabolism and can be measured in serum by radioimmunoassay [14]. It is likely that the serum component resembles monomeric type IV collagen or procollagen which both possess the 7S collagen domain [10, 19]. Thus, the circulating form of 7S collagen presumably reflects the formation and turnover of basement membrane collagen in the body. Induction of diabetes in rats by streptozotocin raised the serum concentration of 7S collagen within 2 weeks [15], and in these animals this rise preceded the morphologically detectable thickening of the capillary basement membrane by several months [11, 18]. It is not known whether increased synthesis or decreased degradation contribute to elevated serum levels.

In the present study, we have measured glomerular basement membrane synthesis in vivo in diabetic and normal rats by injection of labelled proline [2]. Previously described differences in the precursor pool [2, 4] were confirmed by this study and taken into account by determining the specific activity curves of serum proline after injection of the tracer. The possible reasons for the lower specific activity of serum proline in diabetes have been discussed in detail elsewhere [2, 4, 6]. The factors considered so far have been an increased excretion of the radioactive precursor pool [4] or a dilution of the injected tracer. The latter might have occurred either from the enlarged intra- and extravascular volume of diabetic rats [7] or by a larger proline pool resulting from accelerated gluconeogenesis in untreated diabetes [2]. The difference between the specific serum proline activity in diabetic and non-diabetic rats could be abolished in renal tissue by an increased glomerular filtration rate observed in streptozotocin-diabetic rats, which may favour renal exposure to circulating ^{3}H-proline [9]. However, in diabetic rats the specific proline activity of renal cortex is also lowered to the same extent as in serum [2]. The difference in age and duration of diabetes did not influence the purity of basement membrane preparation, as judged by the low phosphorous content and the typical amino acid composition of the isolated basement membranes.

As predicted from a previous study [17], an age-related decrease of basement membrane synthesis was observed in diabetic and non-diabetic groups. However, the formation of hydroxyproline as a specific parameter of collagen synthesis was markedly raised in diabetic rats 18 and 34 days after streptozotocin injection, when compared with metabolically healthy controls. This observation is in agreement with previously published data [2, 4, 6]. Incorporation of proline, as a parameter of total protein synthesis in the basement membrane, was much less elevated in these streptozotocin-diabetic rats. The increase of hydroxyproline activity was distinctly higher than the increase in proline incorporation and most of the latter can be attributed to the proline content of type IV collagen [19]. This shows that the synthesis of the collagen portion of glomerular basement membrane is influenced more by the metabolic disorder than the synthesis of the non-collagenous proteins. However, this alteration of basement membrane metabolism could not be detected in rats with short-term diabetes (4 days), even though blood glucose was increased threefold in these animals.

The concentration of 7S collagen in the sera of diabetic and non-diabetic rats also showed an age-related decrease resembling that observed in basement membrane synthesis. As expected from a previous study [15], the 7S collagen level was markedly raised in diabetic rats compared with controls 18 and 34 days after streptozotocin injection. Furthermore, a positive correlation was found with basement membrane collagen synthesis. A renal insufficiency or exsiccosis of the diabetic animals could account for the serum changes observed. However, the creatinine values were normal and Ilstrup et al. [7] showed previously that diabetic rats did not display a lowered intravascular or extravascular volume when they had free access to water. An increased degradation of the basement membrane with a large release of 7S collagen or related structures is probably not responsible for the elevated levels, since the turnover of basement membranes is slowed down in diabetes [4, 16]. A decrease in the disappearence rate of 7S collagen in diabetes which could lead to raised serum levels was not observed after injection of radiolabelled 7S collagen (D. Brocks, personal communication). Thus, the data indicate that analysis of circulating forms of type IV collagen may be useful in evaluating basement membrane synthesis occurring elsewhere in the vascular system.

A significant correlation was also found for 7S collagen or glomerular collagen synthesis and the blood glucose levels except in the young diabetic animals. With increasing glucose levels, synthesis of basement membrane collagen and serum 7S collagen concentration rose. Previously it has been shown that improvement of metabolic control in diabetic rats by insulin treatment normalized basement membrane synthesis [3, 5] and 7S collagen [15]. Therefore, these findings indicate that the degree of the metabolic dysregulation has an influence on basement membrane synthesis. However, the failure of a corresponding correlation in short-term diabetic rats suggests that the alteration of basement membrane metabolism does not occur simultaneously with induction of diabetes.

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