

# Intracellular Enzymes of Collagen Biosynthesis in Rat Kidney in Streptozotocin Diabetes

Juha Risteli, M.D., Veikko A. Koivisto, M.D., Hans K. Åkerblom, M.D.,  
and Kari I. Kivirikko, M.D., Oulu and Helsinki, Finland

## SUMMARY

The activities of the four enzymes catalyzing intracellular post-translational modifications of the collagen polypeptide chains were assayed in the kidneys of rats with streptozotocin diabetes. When the changes in the four enzyme activities were expressed per milligram of protein in the  $15,000 \times g$  supernatant of the kidney homogenates, there were no changes in any of the enzyme activities at four weeks and only slight increases in the prolyl and lysyl hydroxylase activities at 12 weeks after the induction of diabetes. When the changes were expressed as total enzyme activities per two kidneys, again no changes were found in any enzyme activity at

four weeks, but at 12 weeks significant increases were found in all four enzyme activities, namely prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase, and collagen glucosyltransferase. The data would be consistent with an increased collagen synthesis in diabetic kidneys, but they do not support the hypothesis that there might be specific changes in some of these enzyme activities or in the level of certain posttranslational modifications of the collagen polypeptide chains in this disease.

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The pathology of the renal glomerulus in diabetes mellitus is characterized primarily by thickening of basement membranes and deposition of basement membrane-like material in the subendothelial and mesangial regions.<sup>1-3</sup> The main protein component of basement membranes is a form of collagen, termed type IV, which is rich in hydroxyproline, hydroxylysine, and glycosylated hydroxylysine as compared with the interstitial collagens.<sup>4,5</sup> The glycosylated hydroxylysine in type IV collagen is present almost exclusively in the form of the disaccharide glucosylgalactosylhydroxylysine.<sup>4-6</sup>

The glomeruli of diabetic human patients contain more collagen than those of healthy subjects as measured by hydroxyproline analyses.<sup>7</sup> It has further been reported that the glomerular basement membrane col-

lagen in human diabetics differs distinctly in its composition from that in healthy subjects. The lysine content was decreased, and an equivalent increase in hydroxylysine and hydroxylysine-linked disaccharide units was found.<sup>8,9</sup> However, subsequent reports from other laboratories were unable to confirm these differences in composition.<sup>3,10</sup>

The hydroxyproline, hydroxylysine, and glycosylated hydroxylysine in collagens are synthesized as posttranslational modifications catalyzed by four intracellular enzymes:<sup>6,11-13</sup> prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase, and collagen glucosyltransferase. An increase in collagen glucosyltransferase activity was reported in kidney cortex from alloxan diabetic rats<sup>1</sup> and in lysyl hydroxylase activity in isolated renal glomeruli from streptozotocin diabetic rats.<sup>14</sup> In addition, an early increase in collagen glucosyltransferase activity was found in kidney cortex from a strain of mice that developed genetically transmitted diabetes, but the enzyme activity spontaneously subsided to normal after two months of age.<sup>15</sup>

In the present work the changes in renal collagen synthesis in diabetes were studied further by simul-

From the Department of Medical Biochemistry, University of Oulu, the Children's Hospital, University of Helsinki, and the Department of Pediatrics, University of Oulu, Finland.

Address reprint requests to Juha Risteli, M.D., Department of Medical Biochemistry, University of Oulu, Kajaanintie 52 A, SF-90220 Oulu 22, Finland.

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taneously measuring the activities of the four enzymes catalyzing the intracellular posttranslational modifications. It was assumed that it would thus be possible to find out whether more specific increases take place in some of these enzyme activities.

#### MATERIALS AND METHODS

##### *Animals and the Preparation of Kidney Samples for Assays*

The experimental animals were male Sprague-Dawley rats. They were fed a commercial chow containing 53.0 per cent carbohydrate, 20.9 per cent protein, 4.5 per cent fat, 3.0 per cent fiber, and the usual vitamins and minerals. Two complete experimental series were carried out, the first lasting four weeks (severe diabetes) and the second 12 weeks (moderate diabetes). The age of the rats at the beginning of the experimental series was about three months. The first series contained seven rats in both the control group and the diabetic group, and the second series initially contained six rats in both groups. However, one of the diabetic rats in this series died, and two developed spontaneous remission. Thus, there remained only three rats in this group, and therefore only three control rats were killed.

The diabetes was induced by injecting intravenously streptozotocin in 0.1 M citrate buffer, pH 4.5, as a 3 per cent solution. The dose in the four-week experiment was 60 mg./kg. and that in the 12-week experiment 50 mg./kg. The diabetic state develops with the first-mentioned dose within two days and with the latter dose usually within four days. However, in some cases in the latter group the diabetes is reversible. The animals were not treated with insulin.

Blood specimens were taken from nonfasted animals at the end of the experimental period, and the animals were killed. The kidneys were rapidly removed, decapsulated, immediately frozen in liquid nitrogen, and weighed in the frozen state. They were then stored at  $-70^{\circ}\text{C}$ . until assayed.

The kidneys were homogenized in a Teflon and glass homogenizer (Thomas) at about 1,500 rev./min. for 60 sec. in a cold ( $0^{\circ}\text{C}$ .) solution consisting of 0.2 M NaCl, 0.1 M glycine, 0.1 per cent (w/v) Triton X-100, and 20 mM Tris-HCl buffer, adjusted to pH 7.5 at  $4^{\circ}\text{C}$ .<sup>16,17</sup> The volume of the solution was 9 ml./gm. of kidney. The homogenates were incubated at  $4^{\circ}\text{C}$ . for 30 minutes and then centrifuged at  $15,000 \times g$  for 30 minutes at  $4^{\circ}\text{C}$ . Portions of the supernatant were used for assay of the enzyme activities and the supernatant protein.

*Assays of the enzyme activities.* The incubations in the

assays for prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase, and collagen glucosyltransferase activities were carried out as in our previous studies on liver samples.<sup>16,17</sup> After incubation with prolyl hydroxylase, the reaction was stopped by adding an equal volume of concentrated HCl, and, after hydrolysis at  $120^{\circ}\text{C}$ . overnight, the amount of hydroxy [ $^{14}\text{C}$ ]proline formed was assayed.<sup>18</sup> The reaction with lysyl hydroxylase was stopped by adding 10 ml. of cold acetone, and hydroxy [ $^{14}\text{C}$ ]lysine was measured.<sup>19,20</sup> The [ $^{14}\text{C}$ ]galactosylhydroxylysine or [ $^{14}\text{C}$ ]glucosylgalactosylhydroxylysine formed in the reactions with the two collagen glycosyltransferases was assayed as described by Myllylä et al.,<sup>21</sup> except that the paper-electrophoresis step of the assay procedure was omitted. The effect of this modification on the specificity of the assays with liver samples has been recently reported.<sup>17</sup> Similar studies carried out with kidney samples indicated that over 95 per cent of the product of the glucosyltransferase reaction was recovered in an amino acid analyzer in the position of the glucosylgalactosylhydroxylysine standard, and over 80 per cent of the product of the galactosyltransferase reaction in the position of the galactosylhydroxylysine standard.

*Other assays.* The protein content of the  $15,000 \times g$  supernatants of the kidney homogenates was assayed by the method of Lowry et al.<sup>22</sup> Blood glucose was measured by a glucose oxidase method.<sup>23</sup> All  $^{14}\text{C}$  radioactivity counting was performed in a Wallac liquid-scintillation spectrometer with an efficiency of 85 per cent and a background of 25 c.p.m.

#### RESULTS

##### *Extraction of the Enzyme Activities from the Kidneys*

Recent studies have indicated that all four enzyme activities are associated with membrane structures of the cells in such a way that only part of the enzyme activity can be measured unless the tissue homogenate is treated with some detergent.<sup>16,24-30</sup> In previous studies of lysyl hydroxylase or collagen glucosyltransferase activities in experimental diabetes, the homogenization was carried out with a solution consisting of either 0.25 M sucrose<sup>14</sup> or 0.15 M Tris-acetate, pH 6.8, and 0.002 M 2-mercaptoethanol.<sup>1,15</sup> The extraction of the four enzyme activities with these solutions was therefore compared with that obtained with a solution consisting of 0.2 M NaCl, 0.1 M glycine, 0.1 per cent (w/v) Triton X-100, and 20 mM Tris-HCl buffer, adjusted to pH 7.5 at  $4^{\circ}\text{C}$ .<sup>16,17</sup> The results indicated that the two solutions used in the

TABLE 1

Comparison of three solutions for extraction of the four enzyme activities from rat kidney homogenates

Three kidneys were divided into four similar pieces. One piece of each kidney was homogenized in a solution consisting of 0.2 M NaCl, 0.1 M glycine, 0.1 per cent (w/v) Triton X-100, and 200 mM Tris-HCl buffer, adjusted to pH 7.5 at 4° C.,<sup>16,17</sup> termed NaCl-gly-Triton in this table. The second pieces were homogenized in a solution consisting of 0.25 M sucrose,<sup>14</sup> termed sucrose, and the third pieces in a solution consisting of 0.15 M Tris-acetate, pH 6.8, and 0.002 M 2-mercaptoethanol,<sup>1,15</sup> termed Tris-acetate. The fourth pieces were not used. The homogenization and centrifugation were carried out as described in the text. The enzyme activities were expressed as d.p.m. product formed in the 15,000 × g supernatant obtained from 10 mg. of kidney.

Enzyme activity	Extraction	Product formed (d.p.m./10 mg. of kidney mean ± S.D.)	% of value obtained with NaCl-gly-Triton
Prolyl hydroxylase	NaCl-gly-Triton	9,733 ± 87	100
	Sucrose	3,914 ± 513†	40
	Tris-acetate	2,222 ± 289†	23
Lysyl hydroxylase	NaCl-gly-Triton	245 ± 27	100
	Sucrose	180 ± 21*	73
	Tris-acetate	87 ± 29†	35
Galactosyl-transferase	NaCl-gly-Triton	764 ± 57	100
	Sucrose	447 ± 58†	59
	Tris-acetate	268 ± 40†	35
Glucosyl-transferase	NaCl-gly-Triton	1,488 ± 281	100
	Sucrose	398 ± 78†	27
	Tris-acetate	686 ± 94†	46

\*p < 0.01  
†p < 0.001

previous studies of diabetes extracted considerably less enzyme activity from the kidneys than the solution used in the present study (table 1).

*Effect of Diabetes on the Weights of the Rats and Their Kidneys*

The weight gain of the diabetic rats was retarded, more so in the rats that were severely diabetic (four weeks) than in the rats that were moderately diabetic (12 weeks). However, the weights of the kidneys of the diabetic rats were increased as compared with the normal (table 2). Slightly less protein was found in the 15,000 × g supernatant of the kidney homogenate in the diabetic rats when this value was expressed per gram wet weight of the kidneys. The blood glucose concentration was markedly increased, indicating that all treated rats in the experimental series were diabetic (table 2).

*Changes in the Four Enzyme Activities*

The changes in the four enzyme activities expressed per milligram of protein in the 15,000 × g supernatant of the kidney homogenate are compared with values in the control rats in figure 1. No changes were found in any of the four enzyme activities at four weeks, the largest change (-5.7 per cent) being found in the mean value for collagen galactosyltransferase activity. At 12 weeks a slight increase was found in the prolyl hydroxylase (+25.7 per cent, p < 0.05)

and the lysyl hydroxylase (+14.9 per cent, p < 0.05) activities, whereas no increase, but rather slight decreasing tendencies, were found in the two collagen glycosyltransferase activities (figure 1).

When the changes were expressed as total enzyme activities per two kidneys, again no changes were found in any enzyme activity at four weeks (figure 2).

TABLE 2

Effect of diabetes on the weights of the rats and their kidneys, on the protein content of the 15,000 g supernatant of kidney homogenates and on blood glucose concentration

The age of the rats at the beginning of the experiment was three months, and the initial mean weight was the same in both groups. In the four-week experiments each group contained seven rats, and in the 12-week experiments three rats. Results were expressed as mean ± S.D.

Time and group	Weight of rat (gm.)	Weight of two kidneys (gm.)	Supernatant protein (mg./gm. of kidney)	Blood glucose (mmol/L.)
4 weeks				
Control	337 ± 14	2.05 ± 0.14	131 ± 13	7.2 ± 0.9
Diabetic	238 ± 13†	2.41 ± 0.17†	116 ± 6*	35.6 ± 4.1†
12 weeks				
Control	364 ± 21	1.79 ± 0.08	139 ± 3	5.3 ± 0.6
Diabetic	302 ± 72‡	2.85 ± 0.48*	127 ± 10‡	21.8 ± 3.0†

\*p < 0.01  
† p < 0.001  
‡Not significant.

However, at 12 weeks significant increases were found in all four enzyme activities.

DISCUSSION

The present data contrast with previous reports suggesting that relatively specific increases may take place in diabetic kidneys in some of the enzyme activities catalyzing intracellular posttranslational modifications of collagen biosynthesis.<sup>1,8,9,14,15</sup> No changes were found in any of the enzyme activities at four weeks, at the time when the above-mentioned studies showed increases in the lysyl hydroxylase or the collagen glucosyltransferase activities, and only minor increases were noted in the prolyl and lysyl hydroxylase activities at 12 weeks, when the values were expressed per milligram of protein in the 15,000- $\times$ -g supernatant of the kidney homogenate. In particular, no increase was found in collagen glucosyltransferase activity when the values were expressed on this basis. In one additional series, this enzyme activity was assayed in two diabetic kidneys and two control kidneys at 20 weeks, and again no difference (a decrease by 3 per cent in the mean value) was found. These data do not suggest that any specific increases would take place in the level of hydroxylation of lysyl residues or glycosylation of hydroxylysyl residues in kidney collagen in diabetes, and the data thus

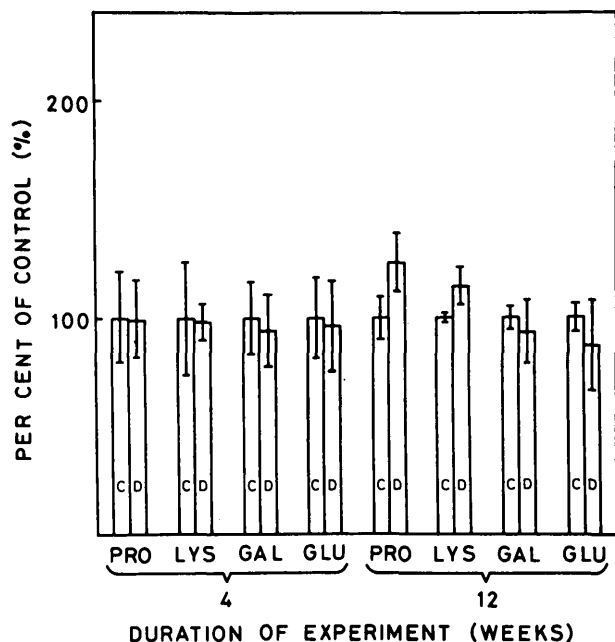


FIG. 1. Changes in the four enzyme activities expressed per milligram of protein in the 15,000  $\times$  g supernatant of the kidney homogenate. The values are expressed as percentages (mean  $\pm$  S.D.) compared with the values in the control rats. Pro = prolyl hydroxylase, Lys = lysyl hydroxylase, Gal = galactosyltransferase, and Glu = glucosyltransferase activity. C = controls, D = diabetics.

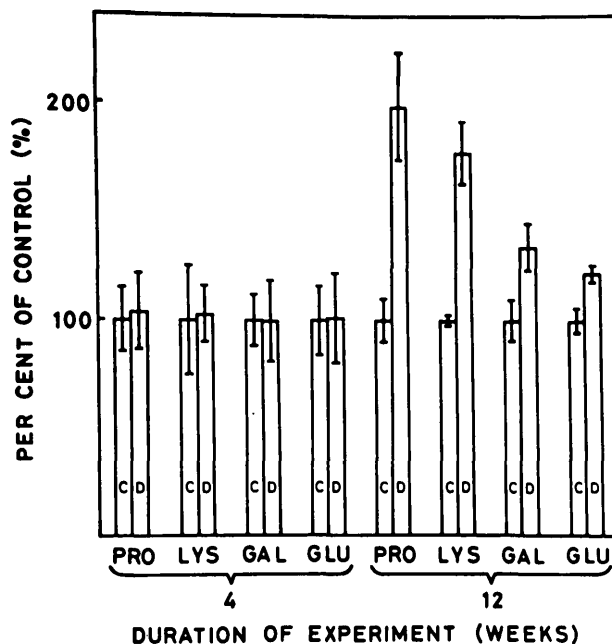


FIG. 2. Changes in the four enzyme activities expressed per two kidneys. The values are expressed as percentages (mean  $\pm$  S.D.) compared with the values in the control rats. For abbreviations, see figure 1.

agree with those of Westberg and Michael<sup>10</sup> and Kefalides.<sup>3</sup>

When the values were expressed as total enzyme activities per two kidneys, a significant increase was found in all four enzyme activities at 12 weeks. It should be noted that the weight of the kidneys was considerably increased, and thus the increased enzyme activities may be related to the synthesis and presence of larger amounts of collagen in the diabetic kidneys. This finding is consistent with the presence of increased amounts of collagen in diabetic glomeruli.<sup>7</sup> It is of interest that larger increases were found in the two hydroxylase activities than in the two glycosyltransferase activities, this pattern being similar to that found during development of experimental liver fibrosis.<sup>16,17</sup>

The diabetes was induced in the present study by streptozotocin, whereas previous studies on collagen glucosyltransferase were carried out either in alloxan diabetes<sup>1</sup> or in genetic spontaneous diabetes.<sup>15</sup> It is not known whether these differences contributed to the differences in the data. An additional difference is that we studied whole kidneys, whereas the previous studies on collagen glucosyltransferase were done with kidney cortex. It seems unlikely that this difference can explain the disagreement in the results, because the cortex comprises about 80 per cent of the weight of the kidneys,<sup>1</sup> and we did not even find any increas-

ing tendency in the supernatant of homogenate from whole kidneys. A major difference between the present and the previous studies is in the composition of the solution used to extract the lysyl hydroxylase or the collagen glucosyltransferase. As demonstrated in this study, considerable amounts of the enzyme activities were lost in the extractions used previously. It may be further noted that in the previous study on lysyl hydroxylase,<sup>14</sup> the increased enzyme activity was found only in the supernatant, whereas a slight decreasing tendency was noted in the particulate fraction.

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