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HISTONES FROM DIABETIC RATS CONTAIN INCREASED LEVELS OF ADVANCED GLYCATION END PRODUCTS

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In a recent report we have demonstrated the in vitro formation of advanced glycation end products (AGEs) on histones in a time and sugar concentration dependent fashion. In the present work we examined histone advanced glycation in vivo. Diabetes was induced in rats by streptozotocin injection and the hyperglycemic state was maintained and surveyed for up to 24 weeks. Diabetic rats showed accumulation of early glycation products in plasma proteins and in hemoglobin. Histones from the liver of diabetic rats showed AGEs levels three-fold higher than those of their age-matched controls. Histone AGEs increased with the duration of diabetes and tended to increase with the age as well. Similar tendencies were apparent in skin collagen. Our data demonstrate that diabetes induces an increase in the accumulation of AGE products on histones. This reinforces the concept that advanced glycation occurs in intracellular proteins and suggests a possible role for intracellular glycation in the increased theratogeny associated with diabetes mellitus. © 1995 Academic Press, Inc.

The Diabetes Control and Complications Trial has unequivocally demonstrated the prevention of complications by a good glycemic control in insulin dependent diabetic patients (1). One of the mechanisms by which sustained hyperglycemia acts, is glycation and its ultimate consequence; advanced glycation. Accelerated modification of proteins by glycation due to chronic hyperglycemia is involved in the pathogenesis of main diabetic complications (2-7). The in vivo demonstration of advanced glycation products (AGEs) in hemoglobin (8) and recently in the cytosol of cells maintained in high glucose (9), highlights the possibility that advanced glycation could occur even intracellularly. As glucose concentration is low within cells, great attention is drawn to glycolytic intermediates as putative candidates for intracellular

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glycation agents. Glucose-6-phosphate, for instance, has been shown to alter DNA mutation rate in prokaryotic strains which accumulate this intermediate(10), and glyceraldehyde-3-phosphate is far more potent as a glycating agent than equimolar amounts of glucose (10). In a recent in vitro study we have demonstrated the formation of AGE products in native histone octamers by incubation with several sugars (11). In particular we have shown that glucose-6-phosphate and fructose, which are elevated in cells from diabetic subjects, have a great potential for generating AGEs in histones. Despite their compact arrangement in nucleosomes, histones are extremely abundant, rich in lysine and usually have the same life span as the cell, all of which make them a target for glycation. In this study we assess the presence in vivo of advanced glycation products in histones from control and streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Animals. Sprague-Dawley rats were used in this study (n=14). Diabetes was induced in 6 of them by a single i.p. injection of 70 mg/kg body wt streptozotocin (STZ). Eight rats, sham injected with saline were used as controls. Animals were given water ad libitum and were fed standard lab chow. All rats injected with STZ had massive glycosuria and hyperglycemia within 24-48 h. Maintenance of the diabetic state was assessed weekly with tailstick glucose. Some of the diabetic animals showed clinical signs of complications: e.g, cataracts and cutaneous infections. After 12 or 24 weeks of hyperglycemia the animals were euthanized and blood as well as liver tissue samples were collected. Glycated plasma proteins and glycated Hb were measured by affinity chromatography on aminophenylboronate columns as previously described (12). Fructosamine was assessed as described previously, with some modifications (13, 14).
Histone purification.Total histones were purified from the liver of diabetic and age-matched control rats as described previously (15). Briefly, nuclei were first isolated from liver homogenates by ultracentrifugation on a sucrose gradient according to the method of Bobel and Potter (16). Washed nuclei were extracted for 1 h with 2 mol/l NaCl, 10 mmol/l Tris/HCl, pH 7.4, containing 1 mmol/l phenylmethylsulfonyl fluoride. The residual nucleohistone was sedimented by ultracentrifugation at 150 000 x g for 16 h in a Beckman XL 70 ultracentrifuge (Bioanalytical System Group, Mississauga, Ont, Canada) and the supernatant, containing total histones was recovered.
Analysis of AGE products in histones. AGE products were measured by their characteristic fluorescence properties, using a Turner 430 spectrophluorometer (AMSCO Inst. Carpinteria CA, USA). Histones were adjusted to 1 mg/ml and fluorescence was measured at excitation maximum of 370 nm and emission maximum of 440 nm for total AGE products and at excitation maximum of 335 nm and emission maximum of 385 nm for pentosidine (17, 18). In both cases fluorescence was expressed in arbitrary units (AU).
Analysis of AGE products in skin collagen. AGE-linked fluorescence in skin collagen was measured as above following the extraction protocol described by Monnier et al (19, 20).
SDS-PAGE was run according to the method of Laemmli, on 15 % gels for histone preparations (21). Equipment employed was Mini Gel from BioRad (Bio Rad Laboratories, Mississauga, Ont, Canada). Gels were stained with Coomasie Brilliant Blue.

Proteins were measured by the micro bicinconninic acid procedure (22)(Pierce, Chromatographic Specialties Inc, Brockville, Ont, Canada). All other chemicals were purchased from Sigma (St Louis MO, USA)
Statistical methods. Data are expressed as mean ± SD. Results were compared by the Student's t test (two tailed) for unpaired data.

RESULTS

Metabolic control of diabetic and control rats

Table 1 summarises the different metabolic parameters of control and diabetic rats. Body weight, as an overall index of metabolic disturbance, was strongly decreased in diabetic animals while their glycemia was extremely high. Glycated Hb, glycated plasma proteins and fructosamine doubled in diabetic animals. Differences between 12 and 24 weeks were not significant. This shows that hyperglycemia, as illustrated by its effect on early glycation of plasma proteins and hemoglobin, reached and remained in maximal steady-state values after the first three months of diabetes. Fluorescence of skin collagen, instead, was higher in diabetic rats and increased from 12 to 24 weeks. A tendency to higher values is also observed for 1 year as compared to 12 weeks control rats. Fluorescence of solubilized skin collagen is an indication of advanced

Table 1. Biochemical follow-up of control and diabetic rats: Evolution of early and late glycation

	12 weeks		24 weeks	
	Controls (n=3)	Diabetics (n=3)	Controls (n=3)	Diabetics (n=3)
Weight (g)	310 ± 25	230 ± 40 [a]	470 ± 35	300 ± 45[a, b c]
Glycemia (mmol/l)	6.6 ± 1.1	28.8 ± 6.6 [a]	5.9 ± 1.0	32.2 ± 5.8[a, b]
Glycated plasma proteins (%)	1.2 ± 0.2	2.5 ± 0.4 [a]	1.3 ± 0.3	2.6 ± 0.4[a, b]
Glycated Hb (%)	4.3 ± 0.6	9.6 ± 1.2 [a]	5.2 ± 0.5	10.3 ± 1.3[a, b]
Fructosamine (mmol/l)	1.8 ± 0.4	3.2 ± 0.6. [a]	2.1 ± 0.5	3.0 ± 0.4[a, b]
Collagen fluo-rescence (AU/mg)	6.3 ± 1.8	12. 5 ± 3.2 [a]	8.6 ± 2.3[a]	18.4 ± 3.9[a, b c]

Results are expressed as mean ± SD. a) p<0.05 vs control rats 12 weeks; b) p<0.05 vs control rats 24 weeks; c) p<0.01 vs diabetic rats 12 weeks.

glycation of this protein (19). Thus, our diabetic rats displayed biochemical and clinical signs of metabolic disfunction, increased early glycation of circulating proteins and increased late glycation of tissular proteins.

Advanced glycation of rat liver histones in diabetic rats

Figure 1 shows AGE fluorescence from liver histones extracted from diabetic and age-matched control animals. Advanced glycation was significantly higher in diabetic animals and increased from 12 to 24 weeks, reaching up to three-fold the levels found in age-matched control animals. When control animals of different ages were compared, a tendency to higher values in histone advanced glycation was also observed between 12 and 52 weeks of age.

Electrophoretic patterns of liver histones from control and diabetic animals

SDS-PAGE of liver histones from diabetic and age-matched control animals is shown in Figure 2. No significant change is apparent in histones bands. This indicates that no cross-linking of proteins due to advanced glycation had yet ocurred.

DISCUSSION

We have recently provided evidence for the in vitro formation of both pentosidine and total AGE fluorescence on histone octamers in a time and sugar

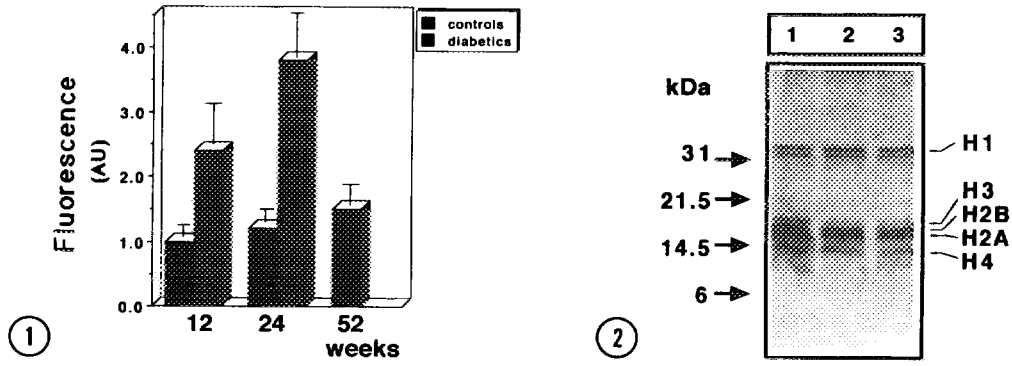

Figure 1. **Advanced glycation of liver histones from control and diabetic rats. Effect of age and length of hyperglycemic state.** Histones were prepared as described in Methods. AGE-linked fluorescence was measured at excitation maximum of 370 nm and emission maximum of 440 nm after adjusting protein concentrations to 1 mg/ml. Diabetics 12 weeks vs controls, $p<0.05$; diabetics 24 weeks vs controls, $p<0.01$; diabetics 24 weeks vs diabetics12 weeks, $p<0.05$. Other differences were not significant.

Figure 2. **SDS-PAGE of histones from control and diabetic rats.** Histones from control or diabetic rats were loaded onto a 15% polyacrylamide gel with a 4% stacking gel. Each lane contained 20 μg protein. Gel was stained with Coomasie Brilliant Blue. 1) diabetic rat, 12 weeks; 2) control rat, 12 weeks; 3) diabetic rat, 24 weeks. No difference in the electrophoretic pattern is observed.

concentration dependent fashion (11). Glucose-6-phosphate or fructose were three times as effective as glucose in generating AGE fluorescence in keeping with data reported for other proteins (23). This highlights their putative role as intracellular glycating agents. It must be remembered that fructose production is increased in diabetic tissues through the sorbitol pathway (2, 4, 24). As lysine residues are constantly available for histone acetylation, they might as well be free and become glycated in the appropriate conditions in vivo. If glycation of histones occurs in vivo, given their long half life, they could also be the site of advanced glycation.

In the light of these facts we have examined in this study the question of histone advanced glycation in vivo. Diabetes was induced in rats by streptozotocin injection and the hyperglycemic state was maintained and surveyed for up to 24 weeks. This was done by frequent glycemia and glycosuria determinations and using long-term indicators of glycemic control: glycated serum protein, fructosamine and glycated hemoglobin levels.

Diabetic rats showed accumulation of early glycation products in plasma proteins and in hemoglobin. The latter reflects the mean glucose values during the entire study while the other two are indications of blood glucose levels for the preceding two weeks (25). Given the catabolic rates of plasma proteins and hemoglobin as well as the slow progress of the glycation reaction by glucose, the observed rises (an overall 100% augmentation) are as expected. They are also in keeping with those found for diabetic patients with poor metabolic control (26-29).

As hepatocyte glucose transporter is not down-regulated by hyperglycemia, liver cells are an appropriate model for the study of intracellular glycation. Histones from liver of diabetic rats as well as from their age-matched controls were isolated. When total AGE linked fluorescence was examined, advanced glycation was found to be increased by up to three-fold. AGE-histone values were higher after 24 than after 12 weeks of diabetes. Histone AGEs tended to increase with the age of the animal as well. Similar tendencies were apparent in skin collagen as previously reported (19, 20, 30-32). It is worthy to mention that one of the rats, which showed both clinical and biochemical signs of extreme metabolic disturbance (small weight, cutaneous infections, bilateral cataracts, and the highest glycated hemoglobin values) had a 6-fold increase in histone AGEs after only 12 weeks of hyperglycemia.

Our data demonstrate that diabetes induces an increase in the accumulation of AGE products on liver histones, complementing our former observation of AGE formation in vitro. Not unexpectedly, the modifications found in vivo, though significant, are milder than those observed in vitro. In particular, no cross-linking of histones was found in vivo, a process previously reported in vitro with high sugar concentrations (11). Our findings have several implications. First, they reinforce the concept that advanced glycation is not a reaction ocurring solely on extracellular proteins as

previously assumed. In particular our data are in keeping with those of Giardino et al (9) who showed a six-fold increase in AGE levels on a basic fibroblastic growth factor when endothelial cells were incubated in vitro in 30 mmol/l glucose for only one week (the mean blood glucose levels of our diabetic rats was within the same range). This modification led to a dramatic decrease in the mitogenic activity of the factor. Moreover, the presence of AGEs in nuclei reported here is in accordance with recent immunohistochemical studies. Indeed, pyramidal neurons from different animals were shown to contain AGE immunoreactivity in their nuclei (33) and in a recent report we have shown that AGE-BSA-gold complex binds cell nuclei matrix with greater intensity in diabetic rats (34).

In conclusion, we provide evidence for an increase in advanced glycation of rat liver histones during diabetes. These data are in keeping with a role for intracellular glycation in the increased theratogeny commonly associated with diabetes mellitus.

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