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Non-enzymatic glycation of elastin

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Non-enzymatic glycation of proteins is one of the key mechanisms in the pathogenesis of diabetic complications and may be significant in the age-related changes of tissues. We investigated the *in vitro* glycation of human aortic α-elastin, and chose and adapted methods for evaluating the degree and kinetics of glycation. α-Elastin was prepared from thoracic aortas of young accident victims and glycated by incubating with different glucose concentrations (25, 50, 75 and 100 mmol/l) in 0.2 M phosphate buffer, pH 7.8 for 30 days, at 37°C. The degree of glycation was measured by three colorimetric methods, *i.e.* Nitroblue tetrazolium, 2-thiobarbituric acid and hydrazine; by aminophenyl-boronate affinity chromatography which determines Amadori products; and by a fluorescence method which determines advanced glycosylation end products. The highest degree of glycation was found on day 3 after the beginning of incubation. Fluorescence, as an index of advanced glycation, consistently increased from days 5 to 24. Investigation of the properties of glycated elastin may help in understanding the importance of this long-lived protein for the age-related changes in tissues and for diabetic complications.

Keywords: advanced glycosylation end products, aging, complications, diabetes, elastin, non-enzymatic glycation

Introduction

Non-enzymatic glycation of proteins in serum and tissues is a pathophysiological consequence of hyperglycemia in diabetes mellitus, and also correlates with aging. Cerami has proposed a hypothesis for the biological role of glucose as a mediator of aging [1]. According to one of the recent, combined theories, age-related changes are induced by the action of free radicals and Maillard reactions [2]. It has been suggested that glycation is the connective link between hyperglycemia and the macrovascular, microvascular and other complications of diabetes mellitus [3]. Products of glycation, that have already appeared, lead to irreversible physicochemical and biological changes of proteins, and in those with a long half-life, to progressive formation of cross-links, *i.e.* to uncontrollable tissue processes, independent of glycemic control [4]. Extensive investigations have been made on the glycation of proteins exposed directly to high glucose concentrations, *e.g.* hemoglobin [5–7], proteins of erythrocyte membrane [8], high- and low-density lipoproteins [9, 10], antithrombin III [11], enzymes [12], insulin [13], collagen [14–18], α-crystallines of lenses [19], fibronectin [20], laminin [21] and peripheral nerve myelin [9, 22].

To date, there have been no investigations on the glycation of human elastin – neither with aging nor in diabetes. Elastin is a long-lived protein, distributed with collagen, and in diabetes is exposed to high glucose concentrations. Connective tissue is thoroughly altered in diabetes. As elastin is a major constituent of it, this raises the question as to whether elastin is subject to glycation and if so what is the role of this process in the pathogenesis of diabetic complications and age-related changes.

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The aim of this study is to investigate the *in vitro* glycation of human aortic α-elastin, and also to adapt methods for assessment of the degree of glycation and to study the kinetics of this process *in vitro*.

Materials and methods

Preparation of human aortic elastin

Human insoluble elastin was prepared from macro- and microscopic unaltered regions of thoracic aortas, obtained from 10 healthy individuals, 18–30 years old who died in accidents, using the methods of Starcher and Gallione [23], Amino acid analysis of the purified elastin showed quantitative similarity to the proteins purified by others [23] and the lack of methionine suggested a low level of contamination. Soluble α-elastin was obtained by the method of Partridge *et al.* [24].

Glycation of α-elastin

α-Elastin (1.33 mg/ml, 10 ml) was incubated with 25, 50, 75 and 100 mmol/l glucose, in 0.2 M phosphate buffer, pH 7.8, containing 0.04% sodium azide, at 37°C for 8 days. Controls were treated under the same conditions, but without glucose. On days 5 and 8 from the beginning of incubation, microbiological testing of samples was carried out to confirm the absence of microbiological contamination.

Methods for assessment of glycation

The colorimetric method for determination of fructosamine with Nitroblue tetrazolium (NBT) [25] was used. The NBT colorimetric procedure is based upon the reducing ability of fructosamine in alkaline solution. Incubated samples (200 μl) were added to 2 ml carbonate buffer, pH 10.8, containing 0.25 mmol/l NBT, at 37°C and the absorbances of the mixtures measured after 5 and 10 min. The incubation times were selected after performing reaction kinetics on two samples. The absorbance differences (ΔE) of every sample were measured three times and the average established. A stabilized solution of glycated human serum—Precimat (Boehringer Mannheim, Mannheim, Germany), containing 344 μmol/l fructosamine, served as a standard. The glycation of the elastin was expressed as mmol fructosamine per g protein.

The colorimetric method with 2-thiobarbituric acid (2-TBA) [26] is based on the hydrolysis of the glycated proteins using oxalic acid at 100°C yielding 5-hydroxymethyl furfural (5-HMF) which reacts with thiobarbituric acid. To avoid the interference by glucose reported by others [27, 28], we dialyzed the samples (1.7 ml) overnight, against physiological saline at 4°C. Aliquots (0.5 ml) of the dialyzed samples were added to 1.5 ml physiological solution and 1 ml 1 mol/l oxalic acid. Hydrolysis was carried out for 4.5 h at 100°C. After cooling to room temperature, the protein was precipitated with 1 ml 5.45 mol/l trichloracetic acid, the mixture centrifuged at 1400 ***g*** for 5 min, and 2 ml of the supernatant added to 0.5 ml 0.05 mol/l 2-TBA. After incubation at 40°C for 40 min, the absorbance was measured at 443 nm. 5-HMF (40 μmol/l; Fluka, Buchs, Switzerland) was used as a standard. The glycation of elastin was expressed as μmol HMF per g protein.

The colorimetric method for determination of glycated proteins with hydrazine [29] is a new method, based on the colorimetry of 2-ketoglucose, which is released from the reaction of glycated protein (ketoamine) with hydrazine. Dialyzed sample (0.5 ml) and hydrazine (4.0 mol/l, 0.2 ml) were heated at 100°C for 0.5 h, 1.2 ml of a 0.02 mol/l solution of phenylhydrazine in 40% (v/v) acetic acid was added and the mixture incubated at 60°C for 1 h. The absorbance of the supernatant, obtained after centrifuging the reaction mixture at 1700 ***g*** for 10 min, was measured at 390 nm. Precimat (344 μmol/l fructosamine) served as a standard. Results were expressed as mmol fructosamine per g protein.

For measurement of glycation using affinity chromatography [30] we adapted a reagent kit (Merck, Darmstadt, Germany) designed for the separation of glycated haemoglobin from non-glycated haemoglobin which uses an agarose column containing covalently bound aminophenylboronic acid (0.5 ml aminophenylboronic acid–agarose) to form a complex with the stable ketoamine, so that this fraction is retained on the column. The non-glycated fraction does not bind and is collected first. The glycated fraction is eluted using an elution buffer containing sorbitol. Following the manufacturers instructions, 100 μl dialyzed elastin sample was added to the column followed by 0.5 ml wash buffer (0.25 mol/l ammonium acetate, 0.05 mol/l magnesium chloride, pH 8.0). After 5 min the non-glycated fraction was eluted with 5.0 ml wash buffer (fraction I). The glycated fraction was eluted with 3.0 ml elution buffer giving fraction II. To determine the concentration of glycated elastin, we used the method of Sedmak and Crossberg [31]. Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 ml 95%

(v/v) ethanol. To this solution was added 100 ml 85% (w/v) phosphoric acid and the resulting solution was diluted with distilled water to a final volume of 1 l. To 1 ml of this solution was added 1 ml of the fractions from the column and the absorbance at 595 nm measured against a reagent blank, after 25 min at room temperature. The concentration of glycated elastin (%) was obtained from the following equation:

$$\%\text{Glyc-elastin} = \frac{3A_2}{5.6A_1 + 3A_2} \times 100$$

A_1 = absorbance of fraction I

A_2 = absorbance of fraction II

The fluorescence (FC) of the glycated elastin was measured as an index of advanced glycation [18]. α-Elastin was incubated with 100 mmol/l glucose for 30 days. For FC estimation, 1.0 ml of the sample was placed in a 1 cm^2 quartz cuvette and fluorescence at 440 nm using excitation at 365 nm was measured. Quininc sulfate (Fluka) 1 μmol/l in 0.1 N H_2SO_4 was used as a standard. The advanced glycosylation end product (AGE) levels were expressed as arbitrary fluorescence units per mg of protein.

Results

Using the NBT method we assessed the fructosamine content of samples incubated with different glucose concentrations on days 1 and 8 from the beginning of the glycation. We previously determined the reaction kinetics for two samples of elastin. The curves obtained suggested that measurements of absorbances should be made after 5 and 10 min (Figure 1). To investigate the reproducibility of the method, coefficients of variation were determined (Table 1). Intra-assay reproducibility (CV) was 4.5% and inter-assay was 5.8%. These results are near to those reported by other authors (6.5%) [25] and show a good reproducibility. The results from glycation, expressed as mmol fructosamine per g protein, are shown in Figure 2. The maximum degree of glycation in all samples with different glucose concentrations was reached on the third day from the beginning of incubation. The highest concentrations of fructosamine were found in the sample incubated with 100 mmol/l glucose, on the third day −0.309 mmol fructosamine/g protein (control, 0.073), *i.e.* fructosamine in the test was 4.2 times higher than that of the control. After the third day lower levels of fructosamine were measured. This was particularly obvious in the sample incubated with 100 mmol/l glucose and may be due to the ketoamine undergoing a series of reactions resulting in the development of AGE products.

Results were obtained by the colorimetric method with 2-TBA. By this method we determined the glycation of samples of α-elastin, incubated with 50 and 100 mmol/l glucose from day 1 to day 8. Intra-assay reproducibility was 8.6% and inter-assay reproducibility was 11.9%. (Table 1). Results were similar to those reported by other authors −9.3% [25] and 13% [26]. The results, expressed as μmol HMF per g protein, are shown in Figure 3. By the 2-TBA method, we confirmed that α-elastin is glycated *in vitro*, as already shown by the NBT method. The maximum degree of glycation was reached on the third day, in the sample with 100 mmol/l glucose, 42.6 μmol HMF/g protein (control 10.0), *i.e.* HMF was increased 4.3 times.

Results obtained by the colorimetric method with hydrazine are not shown. By this method, we determined the glycation of α-elastin, incubated

Table 1. Reproducibility of the colorimetric methods used for determination of early glycation products

n = 20	Colorimetric method with NBT (mmol fructosamine/g protein)		Colorimetric method with 2-TBA (μmol 5-HMF/g protein)		Colorimetric method with hydrazine (mmol fructosamine/g protein)	
	Within-run elastin (25 mmol/l[a])	Between-run elastin (control)	Within-run elastin (50 mmol/l[a])	Between-run elastin (control)	Within-run elastin (100 mmol/l[a])	Between-run elastin (control)
Mean	0.123	0.072	15.02	9.19	0.221	0.072
SD	0.0055	0.004	1.29	1.09	0.0123	0.0056
CV (%)	4.5	5.8	8.6	11.9	5.6	7.8

[a]Elastin was incubated for 4 h with the concentrations of glucose shown.

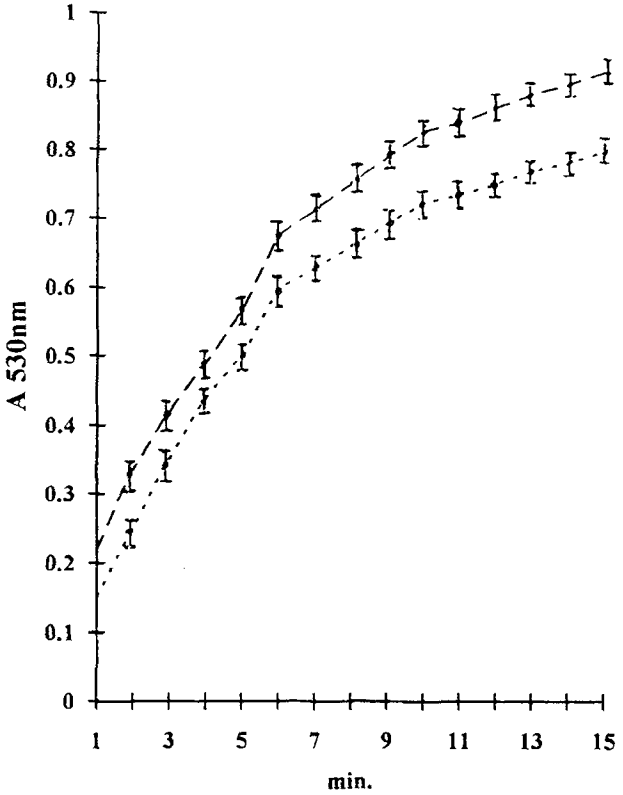

Figure 1. Reaction curves for fructosamine at the 4th hour of incubation: (----) α-elastin incubated with 50 mmol/l; (·····) α-elastin incubated with 25 mmol/l glucose. The data is given as either mean ± SD or as a mean value of three measurements.

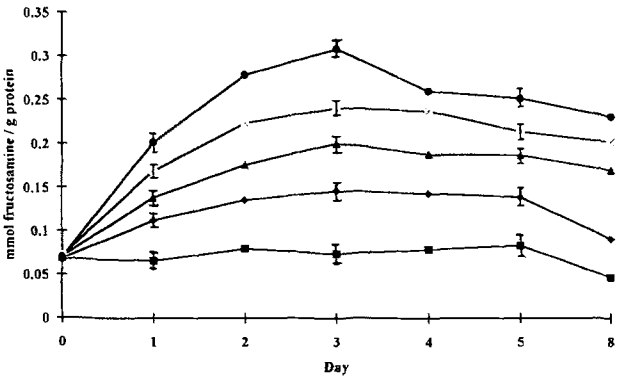

Figure 2. Glycation of α-elastin measured by the NBT method, expressed as mmol fructosamine/g protein in samples with: 25 mmol/l glucose (◆); 50 mmol/l (▲); 75 mmol/l (×); 100 mmol/l (●); control (■). The data is given as either mean ± SD or as a mean value of three measurements.

with 100 mmol/l glucose, on days 1, 3, 5 and 8. Intra-assay reproducibility was found to be 5.6% and inter-assay reproducibility was 7.8% (Table 1). Reproducibility values reported by other authors [29] are 4.4–10.9%. The results, expressed as mmol fructosamine/g protein were: day 1, 0.221; day 3, 0.297; day 5, 0.241; day 8, 0.232 (control, −0.074). On day 3 fructosamine in the test was 4.0 times higher than that in the control.

Glycation of α-elastin (fraction II) after incubation with 100 mmol/l glucose was determined by aminophenylboronic acid chromatography on days 1–5 and 8. We obtained the following results (expressed as a percent of total elastin): day 1, 20.1%; day 2, 24.3%; day 3, 29.7%; day 4, 27.2%; day 5, 25.4%; day 8, 21.0% (control, 9.75%).

Results obtained by FC estimation are shown in Figure 4. The fluorescence of the control was not significantly changed throughout the study. In the test, fluorescence consistently increased from day 5 to 24 and then reached a plateau. On day 24 it was twice that of the control.

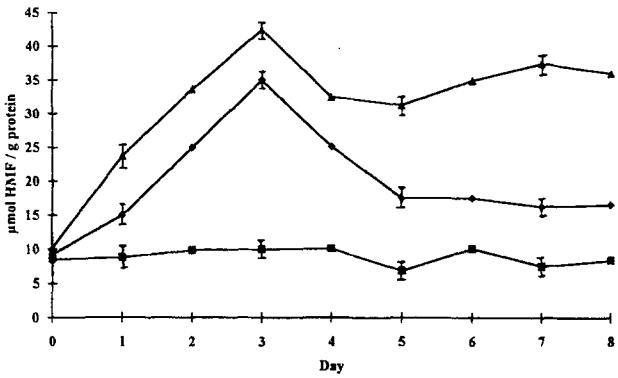

Figure 3. Glycation of α-elastin measured by the 2-TBA method, expressed as μmol HMF/g protein in samples with: 50 mmol/l glucose (◆); 100 mmol/l (▲); control (■). The data is given as either mean ± SD or as a mean value of three measurements.

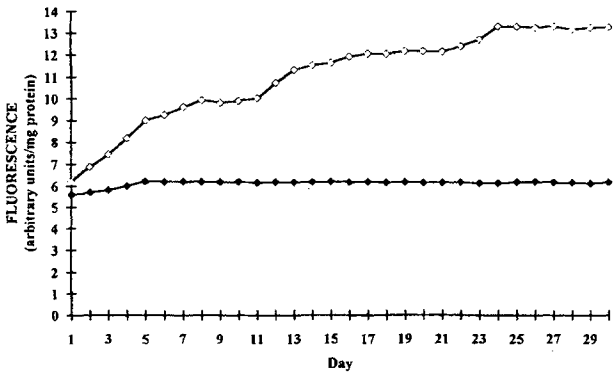

Figure 4. Time course of the changes in α-elastin fluorescence. Time given in days after begining of incubation with 100 mmol/l glucose: test (◇); control (◆).

Discussion

Kinetic studies with other proteins [32] have shown the stimulating effect of phosphate on glycation, which is why we used 0.2 M phosphate buffer in the glycation of α-elastin. Evidence that α-elastin could be glycated, as well as the kinetics and degree of glycation, were established by methods previously used for the determination of early glycation products, *i.e.* three colorimetric and an affinity chromatography method. The fructosamine content has been shown to be dependent on the length of incubation and the glucose concentration, and is a typical feature of early glycation products formed *in vivo*. Similar to events occurring in diabetic tissues [4], we found that fructosamine in all samples reached equilibrium in a given time from the beginning of the glycation. The lower quantities of fructosamine detected after the third day is probably due to the fact that there is a rapid progression from early glycation products to intermediate glycation products and AGEs. The latter are generally stable and irreversible [3]. Their formation from early glycation products is not dependent on the glucose concentrations *in vitro* [33] or the glycemic control *in vivo* [4]. All three colorimetric methods appeared to be applicable to the determination of glycated α-elastin. In the method with NBT, free glucose does not interfere and in the other methods we eliminated interference by dialysis of the samples. The rate of glycation established via the different colorimetric methods was similar. We suggest that the NBT method is the most appropriate for assessment of glycated α-elastin, because it is rapid, it has good reproducibility, free glucose does not interfere and it is easily standardized. The determination and separation of the glycated elastin from the non-glycated form is possible by affinity chromatography. The low percent of glycated α-elastin (≈30%) under high glucose concentrations (100 mmol/l) maybe due to: the special structure of α-elastin which contains small amounts of lysine residues (≈4/1000) and unique amino acids such as desmosine and isodesmosine. The amount of glycation has been found to be limited for other proteins [33].

The results from our fluorescence studies suggest that elastin, like other long-lived proteins, is able to form advanced glycation products. The observed increase in fluorescence may explain the decrease in fructosamine observed in the NBT and 2-TBA glycation methods.

Our future aim is to investigate the antigenic properties of glycated-elastin, compared with those of the relatively poor antigen – unmodified α-elastin. A better understanding of the glycation of elastin is necessary – in the aging process and in diabetes mellitus. We suggest that this is caused by the long half-life of elastin, as well as its distribution in tissues, predominantly altered in diabetes.

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