

Non-Enzymatic Glycation of Human Fibrillin-1

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Key Words

Advanced glycation end products • Fibrillin-1 • Aging process • Non-enzymatic glycation

Abstract

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 isolated and investigated the in vitro glycation of human aortic fibrillin-1. Fibrillin-1 was prepared from thoracic aortas of 9 accident victims distributed in three age groups. The purity of isolated fibrillin-1 was proved. It was glycated by incubating with different glucose concentrations in 0.2 M phosphate buffer, pH 7.4, for 30 days, at 37°C. The degree of early products of glycation was measured by two colorimetric methods, i.e. nitroblue tetrazolium and 2-thiobarbituric acid. Advanced glycation end products (AGEs) were determined by fluorescence measurement. The highest level of early products of glycation was found on day 2 after the beginning of incubation for the fibrillin-1 isolated from the youngest group. Fluorescence in the age groups, as an index of advanced glycation, consistently increased between days 6 and 24. The fibrillin-1 isolated from the youngest group had the highest capacity to form fructosamine and AGEs under glycation in vitro. The capacity of glycation of the 'oldest' fibrillin did not increase significantly during the incubation. Investigation of the properties of glycated fibrillin-1 will help to understand the importance of this long-lived protein to age-related changes in tissues and for diabetic complications.

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Introduction

Reducing sugars such as glucose can react non-enzymatically with protein amino groups (Maillard or 'browning' reaction) to form a variety of structures referred to as advanced glycation end products (AGEs) [1]. These processes are time and concentration dependent such that AGE modifications increase with increasing levels of hyperglycemia and accumulate predominantly in long-lived molecules like extracellular matrix proteins. Over time, the amount of AGE-modified products progressively increase, not only because of persistent glycation, but also because of reduced molecular turnover associated with chronological aging and the resistance of AGE-modified proteins to proteolytic digestion.

The glycation of proteins exposed directly to high glucose concentrations has been studied extensively. Examples include hemoglobin [2–4], interferon- β_{1b} [5], high- and low-density lipoproteins [6, 7], antithrombin III [8], enzymes [9], insulin [10, 11], collagen [12–16], α -crystallines of lenses [17], fibronectin [18, 19], laminin [20], peripheral nerve myelin [6, 21], and IgG and human serum albumin [22].

One important protein group that has not been studied is the fibrillins. The members of the fibrillin family, fibrillin-1, -2 and -3, are extracellular acidic proteins with a high cysteine content and an extended thread-like shape. They are composed of different types of structural modules, including epidermal growth factor (EGF)-like repeats of which the majority have a consensus sequence for calcium binding. Other modules including 8-

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Table 1. Age groups of the purified human aortic fibrillin-1

Age group	Age, years	Aortas, n
I	<5	1
II	28–33	4
III	60–70	4

cysteine motifs, hybrid motifs, and unique domains are interspersed throughout the molecule [23–25]. Fibrillin-1 contains a proline-rich region near the N-terminus that is replaced by a glycine-rich region in fibrillin-2. The fibrillins are found throughout the connective tissue as integral components of extended fibrils. There is expression of a mutant fibrillin-1 in Marfan syndrome.

The objective of this study was: (1) isolation and purification of fibrillin-1 from thoracic aortas, obtained by healthy subjects at different ages; (2) *in vitro* glycation of obtained human aortic fibrillin-1; (3) comparison of different available methods for assessment of early products of glycation and the kinetics of this process *in vitro*, and (4) measuring of the capacity of differently aged fibrillin-1 to form early products and AGEs under glycation *in vitro*.

This work focuses on the investigation of age-related changes in the glycation of human aortic fibrillin-1 in healthy subjects.

Materials and Methods

Materials

Human thoracic aortas were obtained from 9 healthy individuals (following organ procurement) who died in accidents. The samples were distributed in three age groups (table 1). The study was approved by the Ethical Commission of the Medical University of Pleven. Bacterial collagenase (type 1A), phenylmethanesulfonyl fluoride (PMSF), N-ethylmaleimide (NEM), dithiothreitol, Tween-20, CNBr-activated Sepharose 4B, N,N-methylenebisacrylamide, acryl amide, quinine sulfate, keyhole limpet hemocyanin (KLH), diaminobenzidine tetrahydrochloride (DAB), bovine serum albumin (BSA), and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma-Aldrich. Sepharose CL-2B was supplied by LKB. Rabbit anti-human fibrillin-1 antibodies were kindly supplied by Prof. Robert Mecham, Saint Louis, Mo., USA.

Isolation of Microfibrils

Human microfibrillar structures were isolated from normal regions of thoracic aortas using a modification of the method of Kiely et al. [26]. Tissue samples were dissected and minced by

cryohomogenization (with liquid nitrogen). The material was then homogenized in 10 ml of 0.05 M Tris-HCl, pH 7.4, containing 0.4 M NaCl, 0.01 M CaCl₂, 2 mM PMSF, and 10 mM NEM. Bacterial collagenase (type 1A) was added to a final concentration of 0.2 mg/ml, and the digestion was allowed to proceed at 4°C for 48 h with gentle stirring. The digestion was then terminated by the addition of EDTA to a final concentration of 10 mM. The digested tissue homogenates were centrifuged at 10,000 g for 30 min. The supernatants containing solubilized material were designated the low salt extracts. The pellet was resuspended in 30 ml 0.05 M Tris-HCl, pH 7.4, containing 1 M NaCl, 10 mM EDTA, 2 mM PMSF and 10 mM NEM, and then extracted for 48 h at 4°C with gentle stirring before being recentrifuged at 10,000 g for 30 min. The supernatants thus obtained were designated the high salt extracts.

Gel Filtration Chromatography

Low salt and high salt extracts were chromatographed under non-reducing, non-denaturing conditions on a column (1.5 × 82 cm) of Sepharose CL-2B. The column was equilibrated and eluted at room temperature with 0.05 M Tris-HCl, pH 7.4, containing 0.4 M NaCl. Column effluent was monitored at 280 nm using a flow spectrophotometer (LKB Bromma Uvicord S) and 2-ml fractions were collected. The flow rate of the column was 0.4 ml/min.

Affinity Chromatography – Preparation of Human Fibrillin-1

Fibrillin-1 was purified by affinity chromatography. Briefly, antifibrillin-1 antibody was dialyzed against coupling buffer (0.1 M NaHCO₃, 0.5 M NaCl, pH 6.8) and then coupled to cyanogen bromide-activated Sepharose 4B (Sigma-Aldrich) according to the manufacturer's instructions. All coupling steps were conducted at room temperature. The low salt and high salt extracts were applied separately in aliquots of 2 ml to the column and allowed to react with coupled antibody for 30 min. The column was washed well with extraction buffer, and then bound material was eluted with 0.1 M glycine solution (pH 2.8) containing 0.5 M NaCl and 0.1% Tween-20. The eluate was neutralized with 1 M Tris solution. 0.1 M diethanolamine (pH 11) containing 0.5 M NaCl and 0.1% Tween-20 was used as a second elution buffer. The column was regenerated using 0.1 M acetate buffer (pH 4.5) containing 0.5 M NaCl and 0.1% Tween-20, and the purification procedure was repeated. Both acid and basic buffer elution fractions were pooled and dialyzed against distilled water, containing 0.04% sodium azide. The dialyzed material was then dried under vacuum.

Electrophoresis and Western Blotting for Establishing the Purity of Fibrillin-1

The dried fractions obtained by affinity chromatography were analyzed by SDS-PAGE on 5–15% gradient gels under non-reducing conditions. The ratio of N,N-methylenebisacrylamide:acryl amide in the gel was 1:100. Molecular weights were determined by reference to the standards β-amylase, BSA, carbonic anhydrase, and cytochrome c. Gels were stained for proteins using Coomassie brilliant blue. Proteins were transferred to nitrocellulose paper and incubated at room temperature with a 1:500 dilution of anti-fibrillin-1 antibody. Positive reactions were identified after incubation with a peroxidase-labeled secondary antibody from the Vectastain Biotin/Avidin system (Vector Labo-

ratories) according to the manufacturer's instructions. DAB was used as a chromogen.

ELISA for Proving the Specificity of Fibrillin-1 as Antigen

The specificity of pooled aortic fractions was tested by direct ELISA using anti-fibrillin-1 antibody as the primary antibody. The reaction of fibrillin-1 was compared with different heterologous proteins as coating antigens – α -elastin, KLH, BSA, and collagen type IV. The wells of the polystyrene plate were incubated for 2 h at 37°C and overnight at 4°C with either 100 μ l of a solution of dialyzed aortic and skin fractions (10 μ g/ml) dissolved in carbonate buffer, pH 9.6, or the above-mentioned heterologous antigens. After washing with phosphate-buffered saline + 0.05% Tween (PBS-Tween) the wells were blocked with 100 μ l 1% BSA in PBS-Tween for 1 h at 37°C. The plates were then washed and 100 μ l of anti-fibrillin-1 antibody diluted 1:1,000 in PBS was added. The plates were incubated for 1 h at 37°C. After washing with PBS-Tween, wells were incubated with peroxidase-labeled anti-rabbit IgG (diluted 1:3,000 in 1% human serum albumin), and reacted with the substrate solution (0.8 mg *o*-phenylenediamine in 0.05 M citrate buffer, pH 5.0 + 0.01% H₂O₂) for 1 h. The reaction was terminated with 50 μ l 4 M H₂SO₄ and the absorbance was read at 492 nm on automatic micro-ELISA reader Ceres UV900C Bio-Tek Instruments, Inc. Each assay was repeated 3 times and the average established.

Glycation of Fibrillin-1

Fibrillin-1 (1 mg/ml) was incubated at 37°C for 8 days with 25, 50, 75 and 100 mM glucose in 0.2 M phosphate buffer, pH 7.4, containing 0.04% sodium azide. Controls were treated under similar 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.

Assessment of Early Glycation Products (Amadori Products, Fructosamine)

NBT Colorimetric Determination of Fructosamine

The colorimetric method for determination of fructosamine with nitroblue tetrazolium (NBT) [27] was used. The NBT colorimetric procedure is based upon the reducing ability of fructosamine in alkaline solution. Incubated samples (200 μ l) were added to 2 ml carbonate buffer, pH 10.8, at 37°C, containing 0.25 mM NBT, and the absorbance of the mixtures was measured after 10 and 15 min. The incubation times were selected after performing reaction kinetics on two samples. The absorbance differences (ΔE) of each sample were measured 3 times and the average was established. A stabilized solution of glycated human serum, Sero-norm (Merck), containing 314 μ M fructosamine, served as a standard. The glycation of fibrillin-1 was expressed as mM fructosamine per gram protein.

Colorimetric Method with 2-Thiobarbituric Acid (2-TBA)

This method 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 [28]. To avoid interference by glucose, we dialyzed the samples overnight against physiological saline at 4°C. Aliquots (0.5 ml) of the dialyzed samples were added to 1.5 ml physiological saline and 1 ml of 1 M 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 M trichloroacetic acid, the mixture centrifuged at 1,400 g for 5 min, and 2 ml of the supernatant added to 0.5 ml of 0.05 M 2-TBA. After incubation at 40°C for 40 min, the absorbance was measured at 443 nm. 5-HMF (40 μ M; Fluka) was used as a standard. The glycation of fibrillin-1 was expressed as mM 5-HMF per gram protein. Each assay was repeated 3 times and the average established.

Direct Determination of AGEs Formed in vivo

Maillard reaction-related fluorescence (FC) of obtained aortic fibrillin-1 was measured as an index of advanced glycation in 360/450 nm excitation/emission [15, 29] with Corning-EEL fluorimeter. Quinine sulfate 1 μ M in 0.1 N H₂SO₄ was used as a standard. The AGE levels were expressed as arbitrary fluorescence units (AU) per milligram protein.

Investigation of the Capacity of Fibrillin-1 from Different Age Groups to Form AGEs

Fibrillin-1 from groups I, II, and III were incubated with 100 mM glucose in 0.2 M PBS, pH 7.8, containing 0.04% NaN₃, at 37°C for 30 days. Controls of the samples were set on the same conditions, but without glucose. The FC of the samples and their controls was measured from day 1 to day 30 of incubation. The FC difference (sample FC – control FC) for each day was calculated.

Statistical Analysis

The results from the assessment of the formation of early products and AGEs in the three age groups were compared by the Kruskal-Wallis test because of the non-parametric distribution of the data. Median ratings and interquartile ranges (IQRs) were calculated. The statistical package used was SPSS v.13.

Results

To assess the purity of the proteins obtained by affinity chromatography, pooled and dialyzed fractions were analyzed on 5–15% SDS-PAGE gels under non-reducing conditions. Figure 1 shows electrophoretic profiles of the fractions of three age groups. Protein in the extracts had electrophoretic mobilities consistent with the apparent M_r of fibrillin-1 of >300,000. Western blotting was carried out on separated electrophoresis aortic extracts. The bands with M_r >300,000 were stained.

The presence of fibrillin-1 in pooled aortic fractions was also tested by direct ELISA. The reaction of the fibrillin-1 with anti-fibrillin-1 antibody was compared with different heterologous proteins – α -elastin, KLH, BSA, and collagen type IV. The highest extinction values were measured in aortic fractions (fig. 2). The extinction values of the heterologous antigens were close to the control values.

Using the NBT method, we assessed the fructosamine content of samples incubated with different glucose concentrations on days 1–8 from the beginning of the glyca-

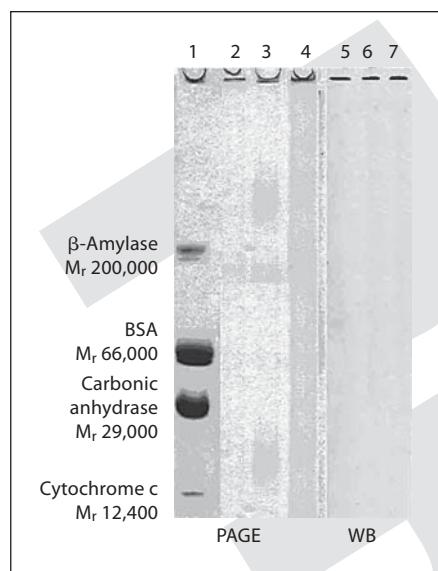


Fig. 1. Electrophoretic analysis (PAGE) and Western blotting (WB) of pooled affinity chromatography fractions. Lanes: 1 = molecular weight markers; 2–4 = aortic fractions obtained by affinity chromatography of low salt extract from age groups I–III; 5–7 = Western blots of affinity chromatography fractions of low salt extract from age groups I–III.

tion. We previously determined the reaction kinetics for fibrillin-1 (group II) incubated with 50 and 75 mM glucose. The curves obtained suggested that measurements of absorbance should be made after 5 and 10 min (fig. 3). To investigate the reproducibility of the method, the coefficients of variation were determined (table 2). Intra-assay reproducibility (CV) was 2.6% and inter-assay was 3.7%. The results from glycation of aortic fibrillin-1, expressed as mM fructosamine per gram protein, are shown in figure 4. The maximum degree of glycation in all samples with different glucose concentrations was reached on the second day from the beginning of incubation. The highest concentrations of fructosamine were found in the fraction isolated from group I incubated with 100 mM glucose, on the second day, 0.233 mM fructosamine/g protein (control: 0.009), i.e. fructosamine in the test was 25 times higher than that of the control. After the second day lower levels of fructosamine were measured. This was particularly obvious in the sample incubated with 100 mM glucose and may be due to the ketoamine undergoing a series of reactions resulting in the development of AGE products. In the sample isolated from group III incubated with 100 mM glucose, the highest concentration was observed on the second day: 0.125 mM fructosamine/g pro-

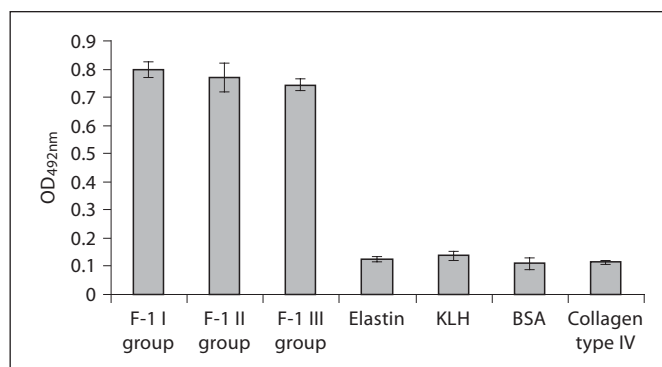


Fig. 2. Direct ELISA for proving the specificity of pooled aortic fractions, compared with several heterologous antigens. Data are shown as the mean \pm SD of three measurements.

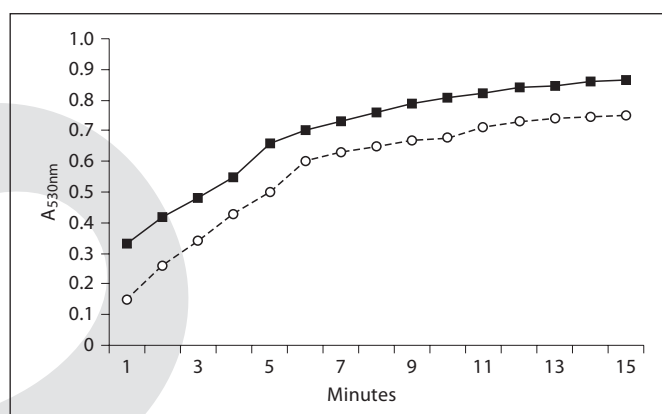


Fig. 3. Reaction curves for fructosamine incorporation into fibrillin-1 (group II) after 4 h of incubation: fibrillin-1 incubated with 75 mM; fibrillin-1 (■) incubated with 50 mM glucose (○). Values are the mean of three measurements.

tein. The Kruskal-Wallis test (table 3a) applied for the samples' concentration of fructosamine formed in vitro during the incubation showed significant differences ($p < 0.05$) between the investigated groups.

Using the 2-TBA method we determined the glycation of samples of aortic fibrillin-1, incubated with 100 mM from day 1 to 8. Intra-assay reproducibility was 6.66% and inter-assay reproducibility was 11.54% (table 2). The results, expressed as mM 5-HMF per gram protein, are shown in figure 5. The maximum degree of glycation was reached on the second day in the fraction from group I: 0.069 mM HMF/g protein compared to 0.04 mM HMF/g protein found in the fraction from group III. The statistical significance of differences between the means of HMF

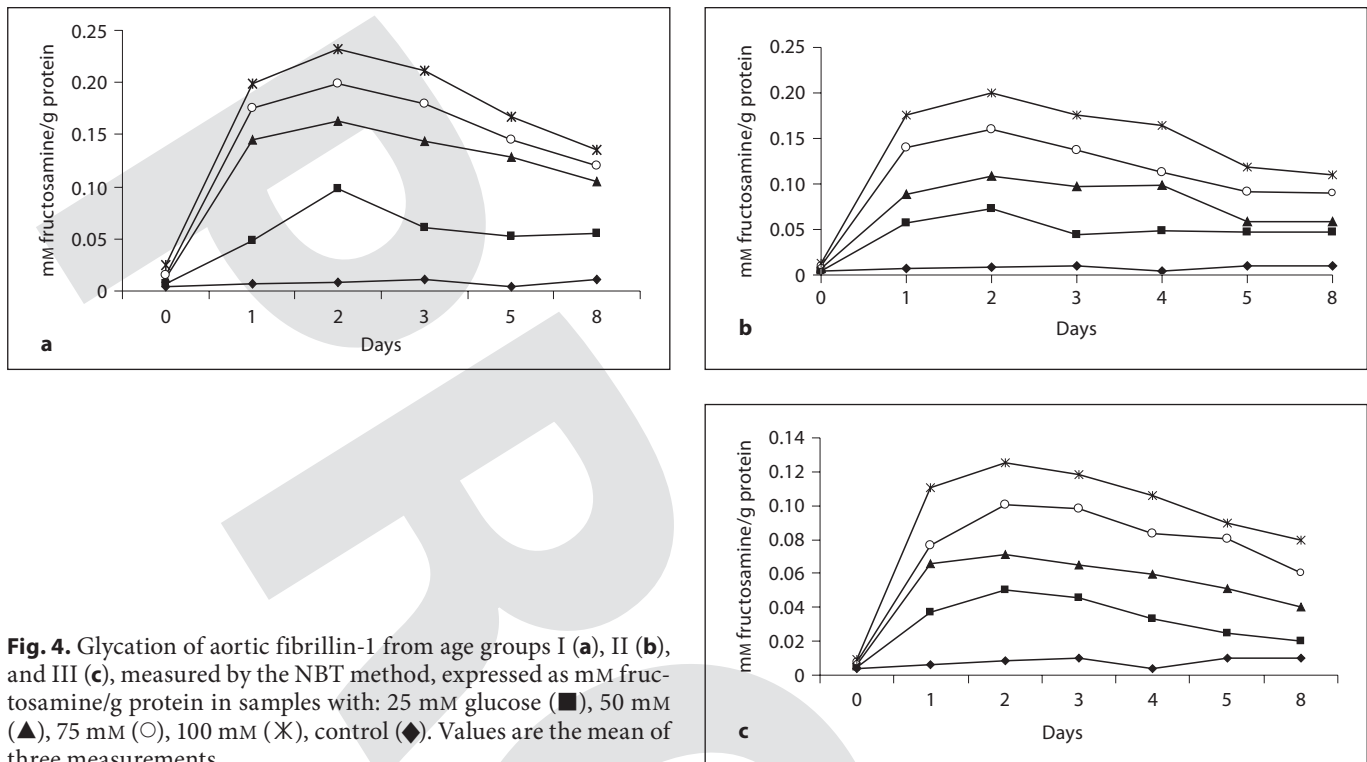


Fig. 4. Glycation of aortic fibrillin-1 from age groups I (a), II (b), and III (c), measured by the NBT method, expressed as mM fructosamine/g protein in samples with: 25 mM glucose (■), 50 mM (▲), 75 mM (○), 100 mM (✱), control (◆). Values are the mean of three measurements.

Table 2. Reproducibility of the colorimetric methods used for determination of early glycation products (n = 22)

	Colorimetric method with NBT mM fructosamine/g protein	Colorimetric method with 2-TBA mM 5-HMF/g protein
Within-run fibrillin-1 (100 mM) ^a	0.123 ± 0.0031	0.054 ± 0.0036
CV, %	2.6	6.66
Between-run fibrillin-1 (control)	0.039 ± 0.0046	0.026 ± 0.003
CV, %	3.7	11.54

CV = Coefficients of variation. Values are means ± SD if not otherwise specified.
^a Aortic fibrillin-1 incubated for 8 days with the concentrations of glucose shown.

content in the samples of the three groups was calculated by Kruskal-Wallis test (table 3b). The content of HMF in the three samples was not significantly different ($p = 0.451$).

Results obtained by direct determination of AGEs formed in vivo are present in AU per mg of fibrillin-1 in figure 6. Sample from the group I showed very low level of FC in vivo: 0.5 AU/mg protein. The highest FC was measured in sample from group III: 6.7-0.5 AU/mg protein. The correlation (Pearson coefficient) between the age and FC of the samples was highly significant ($r =$

0.993, $p < 0.001$). The capacity for in vitro formation of fluorescent AGEs was determined for the 30-day incubation period. Results are present as differences between FC of samples incubated with 100 mM glucose and their controls (without glucose) in figure 7. The fluorescence of the controls was not significantly changed throughout the study. In the samples incubated with glucose, FC consistently increased from day 4 to 15 and then reached a plateau. The highest difference between a sample and control was achieved for group I on day 14 from the beginning of the incubation. The highest difference established

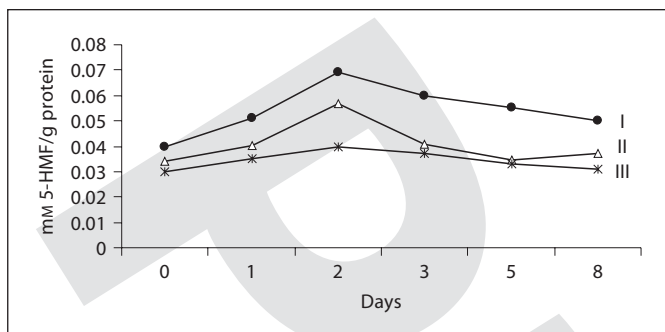


Fig. 5. Glycation of aortic fibrillin-1 from the three age groups (I–III), incubated with 100 mM glucose, measured by the 2-TBA method, expressed as mM 5-HMF/g protein.

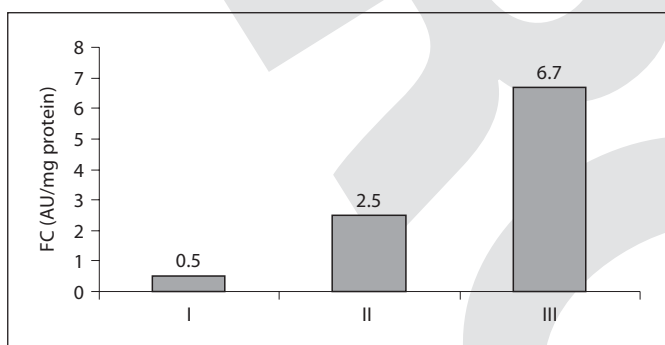


Fig. 6. Direct determination of AGEs formed in vivo. Maillard reaction-related fluorescence (FC), measured for the fibrillin-1, purified from three age groups (I–III).

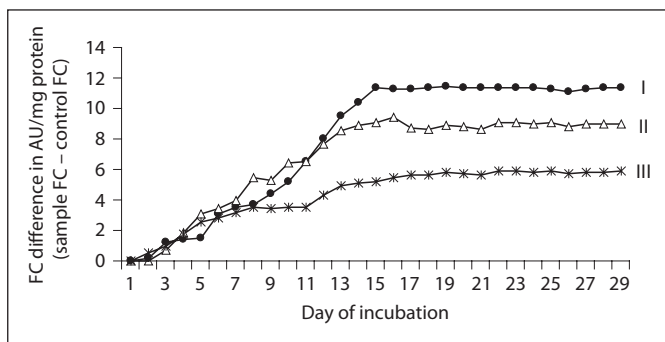


Fig. 7. Difference between fluorescence of fibrillin-1 from the three age groups (I–III), incubated with 100 mM glucose and their controls (without glucose) during the 30-day period of in vitro glycation.

Table 3.

a Kruskal-Wallis test of the results from NBT colorimetric determination of fructosamine in vitro formation

Age group	Median	IQR
I	0.183	0.136–0.211
II	0.17	0.118–0.176
III	0.106	0.08–0.118

Kruskal-Wallis test: $\chi^2 = 6.326$, d.f. = 2, $p < 0.05$.

b 2-TBA colorimetric determination of 5-HMF in vitro formation

Age group	Median	IQR
I	0.053	0.05–0.06
II	0.041	0.035–0.037
III	0.037	0.031–0.035

Kruskal-Wallis test: $\chi^2 = 1.591$, d.f. = 2, $p = 0.451$.

c Formation of AGEs in vitro (applied for the differences between FC of probes and controls)

Age group	Median	IQR
I	11.13	3.73–11.35
II	8.65	5.32–8.96
III	5.23	3.41–5.77

Kruskal-Wallis test: $\chi^2 = 15.667$, d.f. = 2, $p < 0.001$.

for group III was 5.9 on day 23. A significant difference ($p < 0.001$) between the three age groups for the calculated differences between the FC of samples and their controls was established with Kruskal-Wallis test (table 3c).

Discussion

Fibrillins are large (350 kDa) calcium-binding extracellular matrix proteins that form the backbone of the 10–12-nm-diameter microfibrils [23–25]. They serve as a scaffold for the deposition of elastin and form the elastic fiber microfibrils in the extracellular matrix of skin, aorta, lung, muscles and all other dynamic connective tissues [23, 30].

Non-enzymatic glycation is a process in which glucose can directly condense with free ϵ -amino groups of lysine

residues or the N-terminal amino acids of proteins. It is one of the main mechanisms of aging of long-lived proteins. In our previous work, *in vitro* glycation of human aortic elastin was investigated [29] and the formation of early (Amadori) products and late AGEs was demonstrated. To date, non-enzymatic glycation of fibrillins has not been studied. Because the fibrillins are stably associated with elastic fibers, and because of their long half-life, there is a good chance that fibrillins are susceptible to glycation and formation of AGEs. In this study we investigated the *in vitro* formation of early (fructosamine) products and late irreversible AGEs in human aortic ~~and skin~~ fibrillin-1. We chose fibrillin-1 as an object of glycation because, as compared to other fibrillins (fibrillin-2 and -3), it is a major constituent of postnatal connective tissue. Fibrillin-2 is not detectable in postnatal skeletal muscles and blood vessels [31] and, like fibrillin-2, the highest expression of fibrillin-3 occurs in fetal tissue [32].

Low salt and high salt extracts chromatographed under non-reducing conditions on Sepharose CL-2B separates into high molecular aggregates (fraction 1) and small molecules. Fibrillin-1 is present mainly in fraction 1 from both low salt and high salt extracts of aorta and skin. This fraction also contains significant amounts of collagen VI [26]. To purify fibrillin-1, we used immunoaffinity chromatography with cyanogen bromide-activated Sepharose 4B coupled with anti-fibrillin-1 antibody. Because the antibody was polyclonal, we used both acid and basic buffers to elute peptides connected with different subclasses of antibody connected to Sepharose 4B. Acidic and basic fractions were pooled together and analyzed further to assess their purity.

To prove the purity of the obtained fractions these were identified biochemically on the basis of the electrophoretic mobilities and immunologically by Western blotting and ELISA with heterologous antigens. In our initial SDS-PAGE, runs were made using 7.5% gels with 1:30 ratio N,N-methylenebisacrylamide:acryl amide. The fractions, however, failed to penetrate the gel under these conditions. Because of the high molecular weight of the electrophoresed components, it was necessary to lower the cross-linking of the gel in order to facilitate protein penetration into the gel. This was done using a 5–15% gradient gel with 1:100 ratio N,N-methylenebisacrylamide:acryl amide. Under these conditions, the detectable protein moved about 1 cm into the gel (fig. 1). There were no differences in electrophoretic mobility of the fractions isolated from the three age groups.

Kinetic studies with other proteins [33] have shown the stimulating effect of phosphate on glycation; therefore, we used 0.2 M phosphate buffer for the *in vitro* glycation of fibrillin-1. Evidence for the glycation of fibrillin-1, as well as for the kinetics and degree of glycation, was established by methods previously used for the determination of early glycation products, i.e. colorimetric methods. 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 the events occurring in diabetic tissues [34], we found that fructosamine in all samples reached equilibrium in a given time, i.e. 2 days from the beginning of the *in vitro* glycation. The lower quantities of fructosamine detected after the third day is likely due to a rapid progression from early glycation products to intermediate glycation products and AGEs, which are generally stable and irreversible [35]. Their formation from early glycation products is not dependent on the glucose concentrations *in vitro* [36] or the glycemic control *in vivo* [34]. We suggest that the NBT method is appropriate for assessment of glycated fibrillin-1 because it has better reproducibility, there is rapid stabilization of the reaction, free glucose does not interfere at concentrations <144 mM, and it is easily standardized. The TBA method is also applicable to the determination of glycated fibrillin-1, but it takes more time and is complicated because of the daily dialysis, long periods of boiling, and centrifugation of small quantities of samples. There was no statistical significance of differences between the means of HMF content in the samples of the three groups and the reproducibility of this method was lower than in the NBT method.

The different values of measured fructosamine of aortic fibrillin-1 from different age groups could be explained by the different ages of the sample donors. These results are in agreement with our previous investigation of age-related changes in the glycation of human aortic elastin [37] wherein we established the lowering of the capacity of formation of fructosamine and AGEs with aging. With aging there is an increase in the number of lysines and terminal amino acids engaged in formation of early (fructosamine), intermediate (Amadori products), and AGEs. Older tissue possesses less and less free lysine residue ready to interact with glucose.

The results from our fluorescence studies suggest that fibrillin-1, like other long-lived proteins, is able to form AGEs. The observed increase in fluorescence may explain the decrease in fructosamine in the NBT glycation method as the early Amadori products transform into

late irreversible AGEs. The values for the late products reach a plateau because of saturation of free lysine residues.

The accumulation of AGEs in tissue alters the structure and function of long-lived proteins (of fibrillin-1 in particular). Non-enzymatic glycation leads to cross-linking, polymerization, decreased sensitivity to enzyme degradation, etc. As a consequence, new AGE epitopes are formed in and between the chains. In our previous investigation [38], anti-AGE autoantibodies, common for glycated proteins, were studied. Our future aims are to investigate and compare the antigenic characteristics of glycated and unmodified fibrillins. A better understanding of the glycation of fibrillins will help elucidate the role

of microfibrils in the aging process and in pathological conditions.

In our view, these preliminary results contribute to the opinion that non-enzymatic glycation of fibrillin-1 is an age-dependent process. More investigations are necessary to establish the structural and functional impact of fibrillin-1 glycation in physiological and pathological conditions.

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