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Alterations of biochemical and biomechanical properties of rat tail tendons caused by non-enzymatic glycation and their inhibition by dibasic amino acids arginine and lysine

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Summary. The influence of dibasic amino acids arginine and lysine on non-enzymatic glycation of tail tendon fibers from old (900-day-old) and young (61-day-old) rats was investigated in vitro. The biomechanical changes in tendon fibers of young rats after an incubation interval of 7 or 14 days in a glucose solution were abolished by the addition of arginine or lysine (molar ratio amino acid:glucose 1:10). Glucose incorporation into rat tail tendon fibers as well as Amadori product formation was decreased significantly in the presence of the amino acids. The inhibitory effect of arginine was further confirmed by measurement of the amount of ketoamine formed during the glycation reaction using soluble albumin as a protein target. The effective inhibition of non-enzymatic glycation by arginine or lysine suggests their potential use in vivo as a means of controling protein over-glycation.

Key words: Non-enzymatic glycation, arginine, lysine, Amadori product, fructosamine, biomechanical properties.

Non-enzymatic glycation (NEG) of plasma and tissue proteins has been demonstrated in healthy subjects and, to a greater extent, in diabetic patients [1, 2]. Collagenous proteins which govern the biomechanical properties of most tissues are prone to NEG because their turnover rate is very slow [3]. Enhanced glycation of collagen has been reported in diabetes and in animals with experimental diabetes [4–6]. It was also found to increase with age [7–9]. NEG engenders alterations in the biomechanical properties of connective tissue, as exemplified by studies on insoluble tendon collagen [6, 10, 11].

Since changes in the functional properties of proteins caused by NEG are thought to contribute to the well-known long-term complications of diabetes mellitus, the need arises to counteract excessive glycation. Pharmacological intervention, in addition to attempts to achieve an optimal metabolic control, may be of great help in controlling the extent of NEG of proteins. Dibasic amino acids seem to be predisposed as candidates for an in vivo inhibition of NEG, since glycation is initiated by the condensation of glucose with amino groups of proteins to form Schiff's bases. The resulting labile aldimines then undergo a slow Amadori rearrangement to form relatively stable ketoamines [12]. In long-lived proteins such as collagens these Amadori products may condense with other Amadori products or amino acids in adjacent proteins forming so called advanced glycosylated end products [9, 13]. Sensi et al. [14] studied the in vitro inhibition of NEG by lysine, the main amino acid target in proteins for the initial Schiff base reaction. While the pharmacology and diagnostic use of arginine, another dibasic amino acid, for the management of diabetes patients is amply documented [15–17], its role as a potential inhibitor of NEG has not been investigated as yet. In contrast to lysine protein-bound arginine seems to be no target for the aldehyde to Schiff base pathway and, therefore, for NEG in vitro [18]. A recent paper [19] shows, however, that free arginine like lysine is able to form Amadori compounds during long-term incubation with D-sugars. Our objective was to test whether arginine can reduce the effects of NEG on the biomechanical properties of rat tail tendon fibers (RTTF).

Materials and methods

Animals

In our experiments we used tail tendons of two groups of male Sprague-Dawley rats. One group ('young rats') was 61 days old, the other group ('old rats') was 900 days old. The rats were killed with an overdose of pentobarbital.

Preparation of rat tail tendon fibers

The tail tendons were dissected free and single tendon fibers were pulled from the proximal end of the tail avoiding any straining. The tendon fibers were washed extensively in phosphate buffered saline

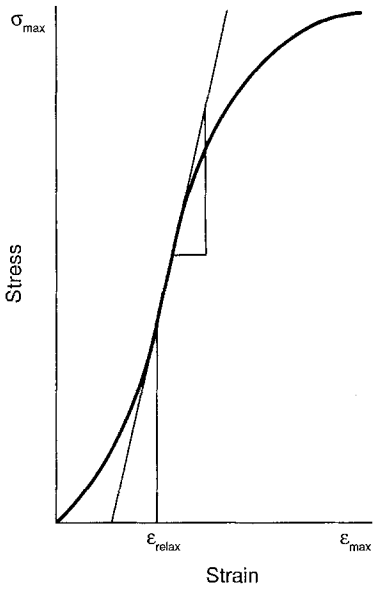

Fig. 1. Typical stress-strain graph of a rat tail tendon fiber. Young's modulus corresponds to the slope at the steepest part of the curve

(20 mmol/l phosphate, 130 mmol/l NaCl, pH 7.2). At the end of the incubation the pH was measured and found unchanged. The cross-sectional areas were in the range 0.02–0.04 mm^2 for young and 0.12–0.65 mm^2 for old rats.

Incubation of rat tail tendon fibers with glucose

Individual RTTF were incubated for 7 or 14 days at 37°C with radioactive glucose (D-[U-^{14}C] glucose, Batch B 191, Amersham International, Amersham, UK) diluted to a specific activity of 8.1 μCi/mmol with unlabelled glucose to give a final concentration of 100 mmol/l in phosphate buffered saline of pH 7.2 containing 0.1% sodium azide as preservative. Incubation volume per tendon fiber was 2.0 ml. The tubes were shaken three times each day. In experiments on the potential inhibitory action of arginine this amino acid was added to a concentration of 10 mmol/l (DL-arginine-hydrochloride, Sigma, St. Louis, Mo, USA). This corresponds to a molar ratio glucose:arginine of 10:1. Analogous experiments were performed using L-lysine. As the controls tendon fibers were incubated in buffer without glucose. Each fiber was cut into two halves of equal length. The pairs of samples were divided into 3 groups: 1) ± glucose treatment for different time intervals, 2) glucose ± arginine and 3) glucose ± lysine.

Incorporation of radioactive glucose

Aliquots of tendons were washed with phosphate buffered saline followed by distilled water (5 times). After lyophilization the weighed samples were solubilized in Soluene 100 (Packard, Downers Grove, Ill. USA), 0.5 ml, at 60°C for 16 h. The samples were added to 10 ml Beckman Ready Value scintillation cocktail (Beckman Instruments, Fullerton, Calif., USA) containing 7.0% acetic acid and counted in a scintillation counter. Glucose incorporation was expressed as μg glucose incorporated per mg dry weight of RTTF.

Non-enzymatic glycation

The amount of glucose bound via ketoamine linkage was estimated as 5- hydroxymethyl-2-furaldehyde (5-HMF), liberated by hydrolysis with oxalic acid. We used a modification of the thiobarbituric acid (TBA) assay of Flückiger and Winterhalter [20]. Standard was pure 5-HMF (Merck, Darmstadt, FRG). RTTF minced with scissors, were washed in phosphate buffered saline, followed by distilled water, and lyophilized. Samples of insoluble lyophilized material were suspended in 1.0 mol/l oxalic acid and homogenized in a glass grinder. The final concentration of tissue suspensions was 5 g/l. After incubating for 4.5 h at 105°C, the samples were allowed to cool in an ice bath. Cold 40% trichloroacetic acid was added to a final concentration of 10%. The mixture was stirred for 20 min and centrifuged at 4000 *g* for 60 min. To aliquots of the supernatants 0.05 mol/l TBA was added to a final concentration of 0.0125 mol/l. After an incubation interval of 15 min at 37°C, the tubes were allowed to stand at room temperature for 20 min before reading the samples at 443 nm. All TBA measurements investigating inhibitory effects of arginine or lysine were performed on one day.

For studies on the in vitro inhibition of NEG of proteins induced by arginine a commercial colorimetric test was used (Roche fructosamine test, Hoffmann-LaRoche, Vienna, Austria). The test principle is based on the reduction of nitroblue tetrazolium by ketoamines at alkaline pH, the resulting formazanes being measured at 550 nm [21]. We applied this test to bovine serum albumin (5.0 g/100 ml). The protein was incubated at 37°C in 100 mmol/l glucose in the presence or absence of 10 mmol/l DL-arginine.

Mechanical tests

Strain-controlled tests were carried out using a computer-aided tensile testing machine, constructed by one of the authors (R. R.). The specimens were loaded at a constant strain rate of 1% per min, which was adjusted by a thyristor controlled gear box-motor. During the tests the specimens were kept in a bath of phosphate buffered saline at 22°C in a thermostated room. The original length (length between the clamps) of all specimens was 10 mm. Because the cross-sectional areas are very small we used a load cell, developed for small loads (range 0.5 mN–10 N). Length and alteration of length was measured using a potentiometer (range 20 mm, resolution 10 μm). According to the function of collagen as main load-bearing element of tendons, we normalized the measured load to the dry weight per unit length, collagen representing 97–98% of tendon fiber dry weight. This definition of 'stress' is reported to ensure the best reproducibility of biomechanical stress-strain behaviour [4, 22, 23]. All specimens were

Table 1. Glucose incorporation and mechanical testing of tail tendon fibers from young rats as a function of glucose concentration. Glucose incorporation and load refer to tissue dry weight

Incubation time (days)	Glucose concentration (mmol/l)	N	[^{14}C] Glucose incorporation (μg/mg)	Load/dry weight/unit length ($mN \cdot \mu g^{-1} \cdot mm^{-1}$)	Time constant (s)	Viscous fraction (%)
0	25	5	–	300 ± 13	78.1 ± 11	18.9 ± 1.9
7		5	0.73 ± 0.1	313 ± 20	63.9 ± 5.9	16.3 ± 0.9
0	50	5	–	213 ± 15	61.3 ± 4.3	22.7 ± 1.6
7		5	1.70 ± 0.3	230 ± 17	80.5 ± 13	17.3 ± 1.8
0	100	5	–	257 ± 21	65.8 ± 5.6	19.7 ± 1.4
7		5	3.78 ± 0.5	338 ± 37	117.0 ± 12[a]	14.3 ± 1.2

Results are expressed as mean ± SEM. [a] $p < 0.05$ values differ significantly between glucose incubated samples and controls

Table 2. Influence of arginine and lysine on glucose incorporation and Amadori product formation in rat tail tendon fibers. All samples were incubated with 100 mmol/l glucose

Age (days)	Incubation time (days)	N	Amino acid added	5-hydroxymethyl furaldehyd (μg/mg tissue dry weight)	[^{14}C] Glucose incorporation (μg/mg tissue dry weight)
61	0	50	–	–	–
	7	26(5)	–	2.24 ± 0.03	3.8
	7	12	arg	1.44 ± 0.04[a]	ND
	14	15	–	2.73 ± 0.05	6.2
	14	7	arg	1.70 ± 0.05[a]	2.9
	14	8(14)	lys	1.55 ± 0.06[a]	3.9
900	0	35	–	–	–
	7	20	–	1.31 ± 0.03	ND
	7	3	arg	1.28 ± 0.05	ND
	14	24	–	1.47 ± 0.04	4.1
	14	12	arg	1.36 ± 0.03	3.4
	14	12(20)	lys	1.32 ± 0.08	2.5

Results are expressed as mean ± SEM. [a] $p < 0.05$ values differ significantly from samples incubated without amino acid addition. N – values in brackets refer to the pooled glucose incorporation samples. ND = not determined

strained to a level of $\varepsilon = 5\%$. After performing relaxation tests at this strain level for a time interval of 10 min the samples were unloaded and then, after a recovery period of 15 min, loaded until the maximum value of stress was achieved.

From this testing procedure one can derive the following parameters (Fig. 1):
- maximum stress, σ_{max}
- extensibility (strain at maximum stress), ε_{max}
- maximum stiffness (maximum Young's modulus), E_{max}
- relaxation time (time needed for stress to decrease to e^{-1} of the original value), τ
- viscous fraction (initial load minus equilibrium load at the end of the relaxation procedure/initial load), æ.

Statistical analysis

When achieving normal distribution and homogeneity of variances, analysis of variances and Student's t-test were performed, otherwise statistical analysis employing Kruskal-Wallis non-parametric test followed by Wilcoxons two sample tests were used. $p < 0.05$ was considered as statistically significant. Results are expressed as mean ± SEM.

Results

Preliminary incubation experiments were performed on RTTF of young rats to determine the glucose concentration range resulting in clearcut changes of the biomechanical properties. From Table 1 it is evident that after one week of incubation marked biomechanical alterations were found only at the highest glucose concentration tested (100 mmol/l). Only changes in the time constant were statistically significant ($p < 0.05$). Glucose incorporation itself was found to be a fairly linear function of glucose concentration.

Based on these preliminary results we investigated the rate of glycation (Table 2) and biophysical parameters (Table 3) as a function of RTTF age and glucose incubation time. Young RTTF show a significantly higher rate of glycation ($p < 0.05$) than old RTTF. As for the biomechanical properties, the maximum stiffness of untreated tendon fibers, normalized to the collagen content, was found to be significantly higher in RTTF of old rats ($p < 0.05$). This is in accordance with the literature [24]. In addition, the extensibility and the time constant of the relaxation phase is higher, whereas the viscous fraction is lower. Glucose incubation for different time intervals (7 and 14 days) generated significant changes only in the biomechanical properties of young RTTF ($p < 0.05$). Thus, we observed a strong increase of maximum stiffness, stress at 5% strain and time constant, accompanied by a decrease of the viscous fraction, after an incubation interval of 14 days in young RTTF.

When adding arginine in a concentration of 10 mmol/l to the glucose solution glucose incorporation was reduced in RTTF of both age groups while formation of reactive carbonyl products was efficiently counteracted only in young RTTF (Table 2). The amino acid inhibited the alterations in stress (at 5% strain), maximum stiffness, extensibility, viscous fraction and relaxation time in young RTTF (Table 3). The biomechanical parameters of the old RTTF with the exception of maximum stiffness were not influenced by arginine addition. Typical normalized relaxa-

Table 3. Influence of arginine and lysine on biomechanical parameters as a function of age of rat tail tendon fibers and different glucose incubation intervals. All samples were incubated in 100 mmol/l glucose

Age (days)	Incubation time (days)	Amino acid added	N	Extensibility (%)	Load/dry weight/unit lenght ($mN \cdot \mu g^{-1} \cdot mm^{-1}$)	Maximum stiffness ($N \cdot \mu g^{-1} \cdot mm^{-1}$)	Time constant (s)	Viscous fraction (%)
61	0	–	50	8.4 ± 0.4	360 ± 16	56 ± 3.0	57.9 ± 2.6	22.1 ± 1.0
	7	–	26	9.3 ± 0.6	340 ± 21	73 ± 5.1	120.0 ± 7.4[b]	13.7 ± 0.9[a]
	7	arg	12	7.2 ± 0.7	300 ± 37	68 ± 5.8	60.6 ± 5.5	24.6 ± 2.3
	14	–	15	9.1 ± 0.7	450 ± 32[a]	113 ± 9.4[b]	123.2 ± 9.9[b]	15.4 ± 1.3[a]
	14	arg	7	6.4 ± 0.8	340 ± 31	69 ± 8.4	52.2 ± 6.2	25.2 ± 3.1
	14	lys	8	5.5 ± 0.6	350 ± 40	107 ± 9.9	53.6 ± 6.4	23.8 ± 2.7
900	0	–	35	16.1 ± 0.9	390 ± 21	84 ± 5.4	85.9 ± 4.9	9.1 ± 2.5
	7	–	20	15.8 ± 1.0	350 ± 28	87 ± 6.8	68.4 ± 7.7	15.1 ± 3.8
	7	arg	3	14.6 ± 3.7	420 ± 77	72 ± 3.1	78.6 ± 9.8	14.0 ± 2.6
	14	–	24	14.6 ± 0.9	420 ± 27	83 ± 5.7	95.2 ± 9.2	7.8 ± 3.5
	14	arg	12	13.7 ± 1.3	390 ± 36	68 ± 6.2	89.8 ± 8.2	6.8 ± 0.6
	14	lys	12	13.9 ± 1.3	380 ± 40	63 ± 6.2	84.4 ± 7.7	5.8 ± 2.3

Results are expressed as mean ± SEM. [a] $p < 0.05$ and [b] $p < 0.01$ values differ significantly from control samples

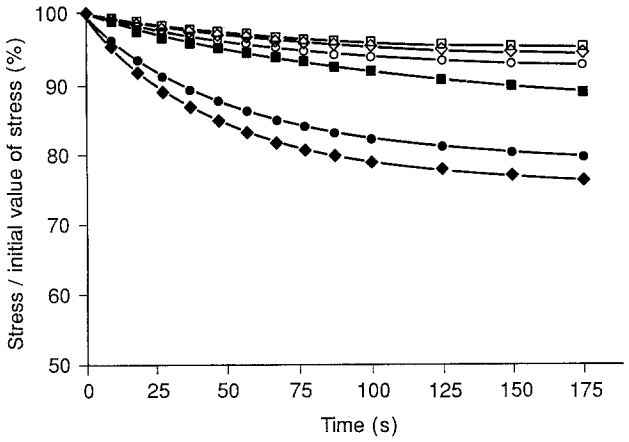

Fig. 2. Graphs of relaxation tests as a function of: ○ tail tendon fibers from old rats; □ after glucose incubation of 14 days; ◇ after glucose incubation in the presence of arginine; ● tail tendon fibers from young rats; ■ after glucose incubation of 14 days; ◆ after glucose incubation in the presence of arginine

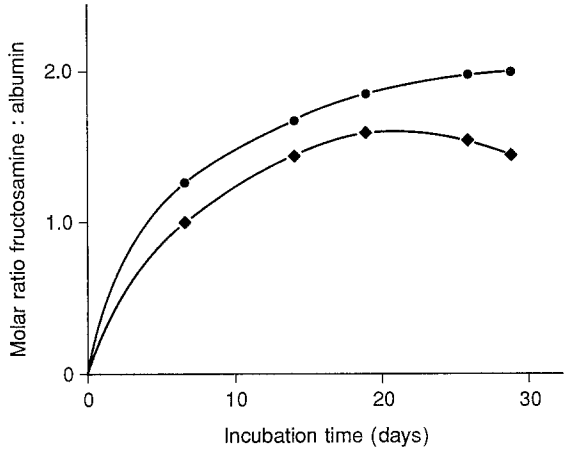

Fig. 3. Fructosamine formation in bovine serum albumin in the presence of 100 mmol/l glucose and the influence of 10 mmol/l arginine (◆ with arginine, ● without arginine). Each point represents mean of two determinations. Error bars are within the symbols

tion graphs showing the influence of glucose treatment and its inhibition by arginine are depicted in Figure 2. Generally, the effects seen by adding lysine to the incubation mixture were comparable to those caused by arginine, with the exception of glucose-induced increase of maximum stiffness which was not counteracted by lysine (Tables 2 and 3).

As an alternative approach to investigating the effect of arginine on the extent of NEG of a soluble protein, we measured the amount of ketoamine formed during in vitro glycation of bovine serum albumin. The results of this experiment showing an approximate 20% inhibition of fructosamine formation after adding arginine are depicted in Figure 3.

Discussion

Biochemical analysis showed that RTTF from the old rats incorporated in vitro significantly less glucose than RTTF from the young rats. This is a consequence of the increased in vivo glycation observed with advancing age as well as the higher degree of crosslinking [4, 7, 25]. Analogous changes were observed in the collagen of patients with diabetes and rats with experimentally induced diabetes [4, 26, 27]. Independent of age RTTF showed a dramatic decrease in glucose incorporation upon addition of arginine to the incubation mixture, pointing to the competitive inhibition by the amino acid of Schiff's base formation between glucose and ε-aminogroups of lysine residues in collagen. The evaluation of the extent of NEG, based solely on incorporation of radioactive glucose is subject to bias and can easily be overestimated [28] (compare Tables 1 and 2, glucose incorporation vs TBA assay). To determine to what extent the further rearrangement of primary glycation adducts to Amadori products is inhibited by arginine, we used two assays that measure ketoamines on intact proteins without interference by Schiff's base products [9], the TBA method and the fructosamine assay. Using the TBA assay which we deem sufficient for comparative ('in one run') determinations we found that arginine significantly inhibits Amadori product formation in RTTF from young rats, while in those of old rats only weak inhibitory effects could be detected. For long-term in vitro tests of the possible inhibitory role of arginine in the NEG of soluble proteins we used the Roche fructosamine test, because in this assay the excess glucose does not interfere.

It is not known, by what mechanism arginine inhibits the initial binding of glucose to collagen and the subsequent Amadori rearrangements. In NEG, glucose is bound to proteins mainly via ε-amino-glycation of lysine residues, a reaction that has been shown to be blocked by soluble lysine [14], which is known to directly attach to glucose via C1 [29] and form reactive intermediates with glucose- 6-phosphate [30]. A recent paper indicates, that also soluble arginine is able to form Amadori products upon incubation with D-sugars [19]. Such a reaction is all the more probable at the large molar hexose excess upheld in our experiments.

According to our biomechanical results the normalized load at 5% strain is independent of the age of the tendons, while maximum stiffness is increased for old RTTF. The observed higher maximum stiffness can be interpreted as a consequence of the higher increase of stress of old RTTF at higher strain values. The extensibility is increased for the old group which may be a result of the larger values of cross-sectional areas of the fibers and has also been reported [31]. After an incubation interval of seven days with glucose we observed in young RTTF a significant decrease of the viscous fraction and a significant increase in relaxation time. This behaviour is in good agreement with the results of Guitton, who found a decrease of the viscosity and the viscoelasticity of glycosylated acid soluble collagen as the main consequence of in vitro NEG [32]. Guitton explains this effect by a decreased hydrophobic intermolecular interaction of collagen molecules. After 14 days, maximum stiffness and stress at 5% strain was also increased in this group.

One of the focal points of this investigation was our interest in the role the dibasic amino acids arginine and lysine might play in modulating the biomechanical changes in-

duced by incubation with glucose. Sensi et al. [14] in their study of the inhibitory effects of lysine on NEG of proteins used the D-isomeric form of the amino acid, relying on the fact that this isomer cannot be incorporated into mammalian proteins. We found, however, that both isomeric forms of arginine or lysine influenced the mode of interaction of glucose with, and the mechanical behaviour of RTTF, subject to our experimental design of in vitro NEG in the same way (data not shown). When incubating the young RTTF with glucose in the presence of arginine for 7 days the above-mentioned glucose-dependent changes in the parameters τ and æ where completely abolished. Additionally, after an incubation interval of 14 days in the presence of arginine the alterations of stress-strain parameters were cancelled. As far as the effects of arginine on old RTTF are concerned, only the maximum stiffness was affected in the sense of a reduction.

In conclusion, this study has shown that in vitro dibasic amino acids can specifically inhibit the non-enzymatic attachment of glucose to RTTF and revert the associated changes in the biomechanical behaviour of the affected tissue. Future studies in animals with artificially induced diabetes must show whether arginine or lysine could be of help in controlling in vivo overglycation of proteins, the hallmark of diabetic hyperglycaemia.

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