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p-Dimethylaminobenzaldehyde-reactive substances in tail tendon collagen of streptozotocin-diabetic rats: temporal relation to biomechanical properties and advanced glycation endproduct (AGE)-related fluorescence

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Abstract

In the present work, pepsin digests of tail tendons from streptozotocin-diabetic rats were found to contain material that reacted rapidly at room temperature with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) to give an adduct with an absorbance spectrum characteristic of the Ehrlich chromogen of pyrrolic nature determined in ageing collagens. A significant correlation of the Ehrlich adduct with tendon mechanical strength and collagen fluorescence characteristic of advanced glycation endproducts was observed. Collagen content of the Ehrlich-positive material was found to be significantly elevated in tendons of diabetic rats compared with age-matched healthy controls. The results indicate that the *p*-dimethylamino-benzaldehyde-reactive pyrrole moieties may contribute to the increased cross-linking of diabetic matrix collagen. Profound inhibitory effect of aminoguanidine was observed, underlining the role of non-enzymatic mechanisms of advanced glycation in pyrrolisation and cross-linking of collagen exposed to hyperglycaemia. It is hypothesised that quantification of the *p*-dimethylaminobenzaldehyde-reactive material in matrix collagen may provide a tissue measure of integrated hyperglycaemia over prolonged periods of time. Further research is to assess the significance of *p*-dimethylaminobenzaldehyde-reactive substances in diabetic collagen tissues and to reveal their relationship to enzyme-mediated physiological pyrrolisation of ageing collagens. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Collagen; Diabetic complications; Ehrlich's reagent; Non-enzymatic glycation; Pyrrole cross-link; Streptozotocin

1. Introduction

In both man and animals, diabetes leads to changes in collagen structure resembling those that occur with increasing age [1,2]. It has been suggested that these effects of diabetes represent an acceleration of the normal ageing process. Since collagen is an important constituent of most of the tissues that are affected during diabetes, modifications of this protein may play a critical role in the etiology of diabetic complications. The postsynthetic modifications of diabetic collagen are attributed mainly to the Maillard reaction, which occurs as a consequence of non-enzymatic glycation [2–7]. Collagen incubated with glucose becomes progressively less soluble and more cross-linked and acquires chromophores and fluorophores with spectroscopic properties similar

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to those of ageing collagen. Oxygen radicals formed during glucose oxidation and glycated protein oxidation may be involved directly in the process of accumulation of advanced glycation endproducts (AGEs) and collagen cross-linking [8,9].

Ageing collagen was shown to contain substance(s) that reacted with *p*-dimethylaminobenzaldehyde in acid solution (Ehrlich's reagent) to give a cherry-pink colour [10]. The *p*-dimethylaminobenzaldehyde-reactive material, s.c. Ehrlich chromogen, also reacted with aryldiazonium salts at pH 1–2 to give coloured products [11]. The collagen-associated Ehrlich chromogen was postulated to be a trisubstituted pyrrole [10,12–14] formed during enzyme regulated physiological maturation of collagen matrices [10,12,15–18]. A cross-linking function of the Ehrlich chromogen in ageing collagen has been stressed. Protein pyrrolisation has been observed also in the processes of non-enzymatic glycation both in model protein–sugar systems under in vitro conditions [13,19–26] and in vivo in experimental diabetes [26–28], and in diabetic patients [22,29].

In the present work, using a model of streptozotocin-induced diabetes in rats, the *p*-dimethyaminobenzaldehyde-reactive material was detected in the rat tail tendon collagen. Time-related relationships of the *p*-dimethyaminobenzaldehyde-detectable material with tendon mechanical strength and with AGE-related fluorescence were evaluated. The effect of the glycation inhibitor aminoguanidine was determined.

2. Materials and methods

2.1. Disease model

The investigation conforms with the Guide for the Care and Use of Laboratory Animals.

Male Wistar rats, 8–9 weeks old, weighing 200–230 g, were used. The animals were of monitored conventional quality and came from the Breeding Facility of the Institute of Experimental Pharmacology (IEP SASc) Dobrá Voda (Slovak Republic). Experimental diabetes was induced by a single i.v. dose of streptozotocin (STZ, 55 mg/kg). STZ was dissolved in 0.1 mol/l citrate buffer, pH 4.5. The animals were fasted overnight prior to STZ administration. Water and food were available immediately after dosing. Ten days after STZ administration, all animals with plasma glucose level >20 mmol/l were considered diabetic and were included in the study. Control animals received 0.1 mol/l citrate buffer.

2.2. Experimental groups

Control and diabetic animals were randomly assigned to three groups: group C, control rats ($n=10$), standard diet; group D, diabetic rats ($n=25$), standard diet; group D/A, diabetic rats ($n=10$), aminoguanidine hydrogencarbonate, 1 g/l in drinking water. During the experiment the animals were housed in groups of two in cages of the type T4 Velaz (Prague, Czech Republic) with bedding composed of wood shavings (exchanged daily). Tap water and pelleted standard diet KKZ-P-M IEP SASc (Dobrá Voda, Slovak Republic) were available ad libitum. The animal room was kept under standard conditions. It was air-conditioned with 10 air changes per hour and the environment was continuously monitored for the temperature of 23 ± 1°C and relative humidity of 40–70%.

2.3. Plasma measurements

Plasma glucose levels were measured at 2-month intervals using the commercial Glucose (Trinder) kit (Sigma, St. Louis, MO).

2.4. Tendon collagen breaking time

At the indicated time intervals of the long-term experiment, the rats were killed, tendons were carefully extracted from skinned tails, washed in saline and preserved deep-frozen under saline. To measure tail tendon breaking time, the fibres were inspected under microscope: single, straight and smooth tendons of an average diameter of 0.15–0.20 mm were selected. Measurements of tendon breaking time were done according to [30] as follows. Weights of 10 g were attached to tendons with surgical suture. Tendons and weights were immersed in 7 M urea solution kept at 40 ± 0.5°C, while the other end was attached to a hook connected to an electric timer. At least four fibres were tested for each animal and results not differing more than 20% were used for calculation of a mean breaking time for each rat. If

larger variations were obtained for the same individual, additional fibres were tested.

2.5. Total pepsin digestion of tendon collagen

Clean tendons (50 mg, wet weight) were cut to small pieces and vortexed in freshly prepared pepsin solution (50 µg/ml in 0.5 M acetic acid, 5 ml) for 24 h at 37°C. Following digestion, the samples were centrifuged at 3000 rpm for 15 min. The clear supernatants containing digested collagen were used for further assays.

2.6. Collagen fluorescence

The samples of pepsin-digested collagen (0.25 ml) were mixed with 2.75 ml of 200 mM phosphate buffer (pH 7.5), and excitation and emission fluorescence spectra were recorded on Kontron SFM-25 to determine wavelength values of maximal excitation and emission. Fluorescence of the samples was quantified at 365 nm excitation and 416 nm emission, relatively to the standard quinine sulfate solution (1 µg/ml). All fluorescence values were corrected for fluorescence of the pepsin blank and expressed per mg of collagen.

2.7. Reaction with p-dimethylaminobenzaldehyde

A small volume (0.2 ml) of 5% (w/v) *p*-dimethylaminobenzaldehyde in 4 M perchloric acid was added to the aliquots (1 ml) of pepsin-digested collagen. Absorbance at 572 nm was measured within 5 min on Hewlett–Packard 8452 spectrophotometer.

2.8. Acid hydrolysis and hydroxyproline assay

Aliquots (1 ml) of pepsin-digested collagen samples were taken up in equal volumes of concentrated HCl (12 M) and incubated in sealed glass ampoules at 110°C for 20 h. Acid hydrolysates were neutralised with NaOH (2.5 M, 4 ml) and mixed with 4 ml of citrate buffer, pH 6. Aliquots of the neutralised samples were used to assay hydroxyproline content according to [31]. Collagen amount in pepsin-digested samples of tail tendons was calculated assuming 14% content of hydroxyproline.

2.9. Materials

Streptozotocin, pepsin and *p*-dimethylaminobenzaldehyde were purchased from Sigma. Aminoguanidine hydrogencarbonate was from Fluka Chemie (Buchs, Switzerland). Other chemicals were of analytical grade quality from local commercial sources.

3. Results

Persistent hyperglycaemia of over 20 mmol/l, measured at 2-month intervals, was recorded in both groups of diabetic animals throughout the whole experiment.

The pepsin digest of collagen from diabetic rats was able to react with *p*-dimethylaminobenzaldehyde under acid conditions to produce a coloured derivative. The spectrum of the coloured product had a maximum at about 572 nm characteristic of the Ehrlich adducts of *p*-dimethylaminobenzaldehyde with pyrroles [10,14,32,33]. As shown in Fig. 1, a time-dependent increase of the characteristic absorbance corresponding to the *p*-dimethylaminobenzaldehyde reaction product in tail tendon collagen was observed during the course of the 8-month experiment. In the fourth and eighth month, significantly higher values of the Ehrlich-positive material were obtained in di-

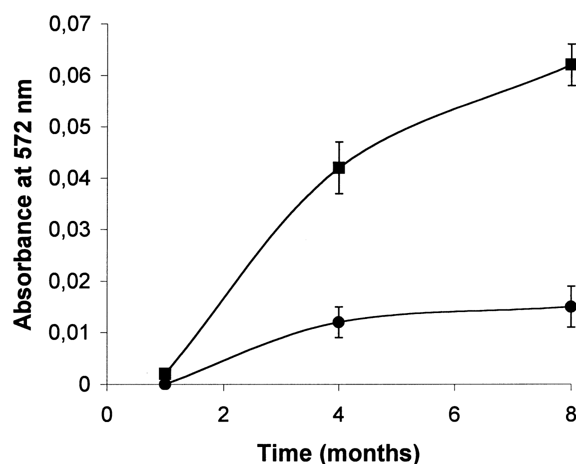

Fig. 1. Ehrlich adduct absorbance in rat tail tendon collagen tissue as a function of time. Absorbance was normalised to collagen concentration of 10 mg/ml in 0.5 cm cuvette. (●) Control animals (group C, $n=8$); (■) untreated diabetic animals (group D, $n=17$). Results are mean values ± S.E.M.

abetic animals when compared with those of control healthy rats ($P < 0.001$).

The breaking times of collagen tendons isolated from rat tails were determined as a measure of collagen cross-linking. As shown in Fig. 2, the tendon breaking time values correlated positively with the Ehrlich adduct absorbance ($r = 0.84$). The cross-linking marker values for diabetic rats were found to be significantly higher than those for age-matched control animals ($P < 0.001$).

Collagen obtained from tail tendons of diabetic animals showed increased fluorescence with excitation and emission maxima of 365 and 416 nm, respectively. Collagen-linked fluorescence was quantified as a measure of advanced glycation. As shown in Fig. 2, AGE-related fluorescence of tendon collagen correlated positively with the Ehrlich adduct content ($r = 0.85$), the values for diabetic animals again being significantly higher than those for age-matched controls ($P < 0.001$).

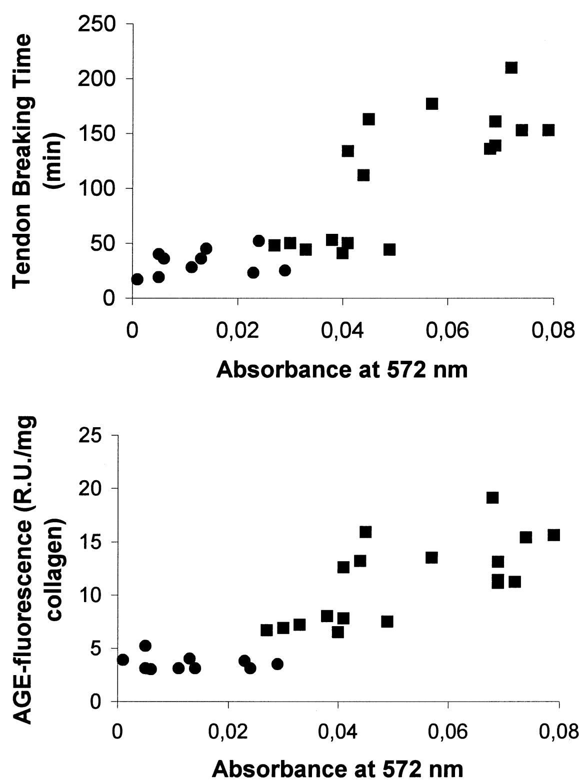

Fig. 2. Relationships of Ehrlich adduct absorbance in rat tail tendon collagen tissue to tendon breaking time and to AGE-related collagen fluorescence ($r = 0.84$, $r = 0.85$, respectively). The results were obtained in the fourth and eighth month. (●) Control animals (group C); (■) untreated diabetic animals (group D). $P < 0.001$, diabetic animals vs. age-matched controls for each parameter measured.

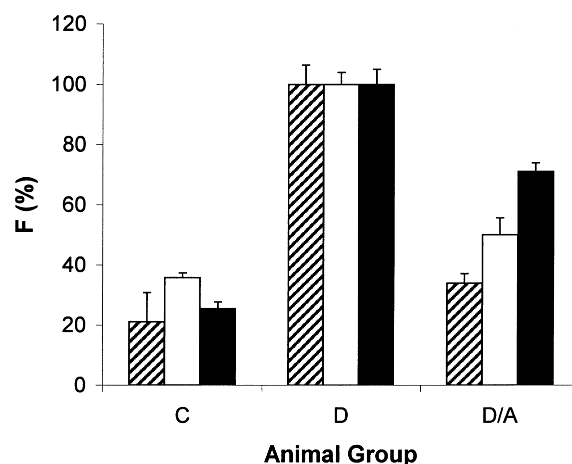

Fig. 3. Changes of tail tendon collagen in streptozotocin-diabetic rats. Effect of aminoguanidine. F: values represent Ehrlich adduct absorbance (shaded bars), tendon breaking time (white bars) and AGE-related fluorescence (black bars) expressed as percentage of the respective values obtained for untreated diabetic animals (group D). C, control rats; D/A, diabetic rats treated with aminoguanidine. Results were obtained at the end of the 8-month experiment and are expressed as mean values ± S.E.M. ($n = 6$–17).

The glycation inhibitor aminoguanidine significantly inhibited changes of all three parameters evaluated (Fig. 3; $P < 0.001$, D/A vs. D). The most profound inhibition was observed for the Ehrlich adduct.

4. Discussion

The results obtained in this study showed pepsin digest of tail tendons from diabetic rats to contain material that reacted rapidly at room temperature with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) to give an adduct with an absorbance spectrum characteristic for the Ehrlich chromogen of pyrrolic nature determined in ageing collagens [10,15–18]. Analogous spectra were obtained in reaction of *p*-dimethylaminobenzaldehyde with other pyrrole derivatives [14,32,33]. In comparison with age-matched healthy control animals, the diabetic rats were found to contain significantly elevated levels of the Ehrlich adduct. Using the extinction coefficient of 35 000 $M^{-1} s^{-1}$ calculated by Hidalgo et al. [14] for the

Ehrlich adduct of synthetic pyrrolylnorleucine, content of the Ehrlich-positive material in the rat tail tendons could be estimated as 350 and 86 pmol/mg of collagen for diabetic and control rats, respectively, at the eighth month of the experiment. The present findings are in accordance with protein pyrrolisation caused by elevated levels of glucose under near physiological conditions in vitro [13,19–26], in vivo in experimental diabetes [26–28], and in diabetic patients [22,29]. Non-enzymatic processes of advanced glycation may be implicitly involved in the formation of pyrrole structures in collagen, e.g., via chemical reaction of sugar-derived intermediary α dicarbonyls with protein amino acid residues [13,25].

Tendon breaking time, often used as a measure of collagen cross-linking [30,34–37], was significantly increased in diabetic rats in the fourth and eighth month of this experiment. The correlation ($r = 0.84$) of the tendon breaking time with the content of the Ehrlich-positive material underlines, in agreement with literature data [10,15–18], the cross-linking function of this, most likely, pyrrolic moiety. However, the mechanism of cross-link formation and the overall contribution of *p*-dimethylaminobenzaldehyde-reactive products to the cross-linking of collagen remains still uncertain. Apart from the non-enzymatic Maillard reaction, the diabetic collagens may be modified by at least two additional processes: lysyl oxidase mediated physiological cross-linking and by interactions with products of lipid peroxidation. The interrelationships between these and possibly other postsynthetic modifications in diabetes mellitus remain unclear. Lysyl oxidase activity was found increased in kidneys [38,39], lungs [40] and skin [41] of diabetic subjects. Other authors [6,36, 42,43], however, came to the conclusion that the physiological lysyl oxidase dependent cross-links would hardly explain the changes in physical strength of tendon fibres observed in diabetic animals. In view of the earlier findings that aminoguanidine itself does not influence the formation of lysyl oxidase dependent reducible cross-links [44,45], the inhibitory effect of aminoguanidine observed here stresses the role of non-enzymatic mechanism(s) in collagen pyrrolisation and cross-linking. Apart from the pyrrole-like substances yielding the Ehrlich adduct, other products of non-enzymatic advanced glycation, particularly pentosidine [6,37], as well as the products of lipid peroxidation [46,47], may participate in the observed increase of tendon breaking time in diabetic rats.

Collagen obtained from tail tendons of diabetic animals showed increased fluorescence with excitation and emission maxima characteristic of products of advanced glycation [48]. On recording the emission spectra, a broad band was obtained, which is likely to represent a mixture of chemical compounds. Considering the correlation of the Ehrlich adduct absorbance and AGE-related fluorescence (see Fig. 2), contribution of the *p*-dimethylaminobenzaldehyde-reactive moieties to collagen-linked fluorescence seems to be considerable. The inhibitory effect of aminoguanidine indicates the participation of mechanisms related to non-enzymatic advanced glycation.

On balance then, the results obtained in this study indicate that the presence of *p*-dimethylaminobenzaldehyde-reactive moieties, most likely of pyrrolic nature, in diabetic connective tissues may be at least partially responsible for increased cross-linking of diabetic matrix collagen. The marked inhibitory effect of the glycation inhibitor aminoguanidine is strongly indicative of the involvement of non-enzymatic glycation mechanisms in collagen pyrrolisation. We hypothesise that quantification of the Ehrlich-positive material might provide a tissue measure of integrated glycaemia over prolonged periods of time, yet further research is to assess the significance of *p*-dimethylaminobenzaldehyde-reactive substances in diabetic collagen tissues and to reveal their relationship to enzyme-mediated physiological pyrrolisation of ageing collagens.

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