Collagen Aging In Vitro by Nonenzymatic Glycosylation and Browning

ROBERT R. KOHN, ANTHONY CERAMI, AND VINCENT M. MONNIER

SUMMARY

Aging and diabetes mellitus are associated with crosslinking and nonenzymatic glycosylation of collagen. Incubation of tendon fibers with reducing sugars results in increased breaking time in urea similar to that seen in aging, and in nonenzymatic glycosylation and browning. Effect of a sugar is proportional to the amount of sugar available in the open chain form. The increase in breaking time correlates with the appearance of chromophores characteristic of crosslinked browning products. Collagen altered by nonenzymatic browning may play a role in some age-like major complications of diabetes. DIABETES *33*:57–59, January 1984.

Some of the serious and characteristic complications of diabetes mellitus, including arteriosclerosis, resemble aging processes and occur in collagen-rich tissues.[1–5] Aging of collagen is associated with increased crosslinking as manifested by decreasing solubility,[6] increasing stiffness,[7] and increasing resistance to enzymatic digestion,[8] and many age-related processes in collagen have been found to be accelerated in diabetes.[6,9–11] Nonenzymatic glycosylation has been demonstrated in collagen from several sites in human and experimental diabetes[10,12–15] and has been shown to increase with age in human collagen.[10]

Glycosylation is initiated by a reducing sugar such as glucose reacting with amino groups of proteins. Reaction rate increases with increasing blood levels of sugar.[16,17] The reaction proceeds by an Amadori rearrangement through the formation of a ketoamine that can be determined, and, then, by the so-called late Maillard or browning reaction, to the formation of crosslinked pigmented and fluorescent compounds.

Lens proteins, other relatively inert macromolecules, undergo glycosylation with age, and incubation of lenses with sugars has been shown to lead to the formation of fluorescent and pigmented compounds characteristic of nonenzymatic browning reactions.[18,19] We incubated tendon fibers with sugars to determine if nonenzymatic glycosylation of collagen would occur, progress to browning, and result in crosslinking similar to that occurring with age.

MATERIALS AND METHODS

Use was made of the breaking time of tendon fibers in 7 M urea at 45°C, a very sensitive measure of collagen and of animal age that depends on density of collagen crosslinking.[20,21] Tail tendon fibers from a single 11–13-wk-old Sprague Dawley rat were used for each experiment. Each breaking time was determined for three fibers in an apparatus and by methods described for use with both mouse and rat tail tendons.[20,21] An apparatus for 20 simultaneous breaking time determinations was constructed using a precisely regulated water bath, electric timers, and snap microswitches (Allied Electronics, Willoughby, Ohio, cat. nos. 765-0632, 829-3002).

Rate of nonenzymatic glycosylation of proteins depends on the amount of a sugar present in the open chain form, and perhaps is affected by bonding of the sugar to specific protein sites.[22–25] Sugars used were ribose, glucose-6-phosphate, and glucose. Ribose is most effective and glucose least effective in glycosylation.[25] Tendon fibers were incubated at 37°C in 0.1 M solutions of the sugars in phosphate-buffered 0.15 M NaCl, pH 7.4. A small drop of chloroform was added to the solutions, and corks were moistened with toluene to inhibit bacterial growth. In one experiment fibers were incubated at 37°C in 0.1 M sodium cyanate for 3 h, washed, and then incubated with glucose-6-phosphate. Cyanate reacts specifically with amino groups of proteins[26] and might inhibit glycosylation of amino groups and subsequent age-like crosslinking.

From the Institute of Pathology, Case Western Reserve University, Cleveland, Ohio (R.R.K., V.M.M.), and The Laboratory of Medical Biochemistry, Rockefeller University, New York, New York (A.C.).
Address reprint requests to Dr. R. R. Kohn, Institute of Pathology, Case Western Reserve University, Cleveland, Ohio 44106.
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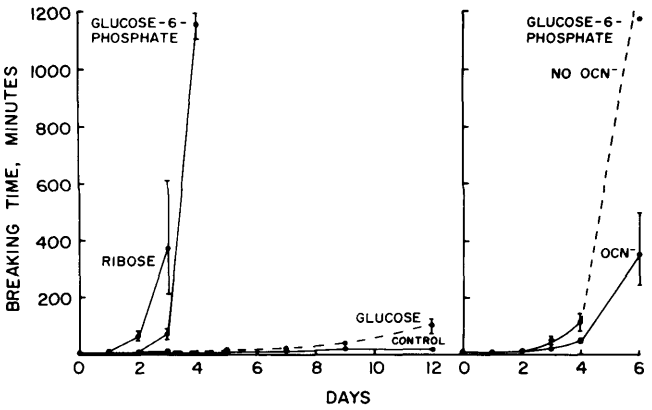

FIGURE 1. Left: Breaking time of rat tail tendon fibers in 7 M urea at 45°C after incubation for various times in 0.1 M solutions of ribose, glucose-6-phosphate, glucose, or phosphate-buffered saline (control) at 37°C. Vertical line represents range of three determinations, with curve drawn through mean. Right: Breaking time of tendon fibers incubated in 0.1 M glucose-6-phosphate for various times, with and without prior incubation in 0.1 M sodium cyanate for 3 h at 37°C followed by thorough washing. Fibers not preincubated with cyanate did not break by 1180 min after 6 days of exposure to the sugar; the experiment was terminated at that time.

After incubation, tendon fibers were washed, minced, and completely digested by 24-h incubation at 37°C in 0.1 M $CaCl_2$ and 0.02 M Tris-HCl, pH 7.55, containing bacterial collagenase (Worthington, CLSPA) with an enzyme:substrate ratio of approximately 1:10. The small amount of noncollagenous material was removed by centrifugation; collagen in supernatant solutions was determined by a modification of the method of Stegemann and Stalder.[27] Samples were diluted to contain 5 mg of collagen digest and 1 mg of enzyme per ml, and nonenzymatic glycosylation was determined by a thiobarbituric acid method,[28] employing fructose as a standard. Hydroxymethyl furfural (HMF) is determined by this method. The absorption spectra of material released by oxalic acid treatment of collagen reacted with glucose and glucose-6-phosphate, and reacted with thiobarbituric acid, were found to be identical. Absorbance and fluorescence spectra of the digests were obtained by procedures used to characterize nonenzymatic browning products in lens proteins.[19]

RESULTS

Tendon fibers incubated with sugars showed increased breaking time similar to that occurring with aging. Ribose was more effective than glucose-6-phosphate and both were much more effective in increasing breaking time than glucose, the latter causing a slow rise in breaking time (Figure 1). There was a lag period of 2–3 days incubation before the abrupt increase in breaking time seen in tendons incubated with ribose and glucose-6-phosphate. Prior incubation with cyanate caused inhibition of the effect of glucose-6-phosphate on breaking time (Figure 1), supporting the notion that this effect is mediated by covalent modification of free amino groups.

Absorption spectra of digested collagen from tendon fibers incubated for 19 days with the three sugars are shown in Figure 2. An increase in absorption from 300 nm to 450 nm was observed with all sugars, indicating the presence of several chromophores. Their concentration correlated with effect of the sugars on tendon fiber breaking time. Collagen of tendon fibers incubated with the rapidly reacting sugar, glucose-6-phosphate, showed increases from the beginning of incubation in nonenzymatic glycosylation and fluorescence (Figure 3). Glycosylation reached a maximum at around 6 days, while fluorescent products increased linearly for the first 20 days. There was a lag of about 3 days in the formation of yellow chromophores (Figure 3). Time of chromophore formation correlated with the increase in breaking time of tendon fibers incubated in glucose-6-phosphate (Figure 1).

DISCUSSION

After this study was concluded, a report appeared describing increased tail tendon stability in diabetic rats, and following incubation of tendon fibers with glucose.[29] These data, the data presented here, and previous reports support the following sequence: In aging, and at a greater rate in diabetes, sugars nonenzymatically glycosylate amino groups of collagen. Rate of glycosylation is proportional to the availability of the open chain form of sugars. Fortunately, as pointed out by Bunn and Higgins,[25] glucose, the major extracellular sugar, glycosylates very slowly. The ketoamine resulting from an Amadori rearrangement reacts further to form fluorescent compounds that then yield pigmented crosslinked compounds. Composition of the latter would vary with the kind of sugar involved. This sequence is similar to that reported for the browning or Maillard reaction in stored food.[30]

Although proof is lacking, it is possible to explain all of the age-like changes in collagen properties in diabetes on the basis of crosslinking associated with nonenzymatic browning; such crosslinks plus other types would be expected in natural aging. Overall extent of human diabetic complications has been correlated with nonenzymatic glycosylation

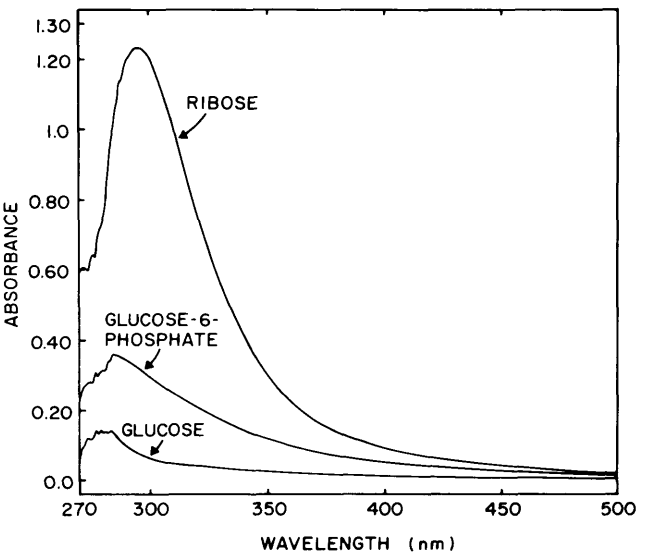

FIGURE 2. Absorption spectra of collagen digest, 5 mg/ml, from rat tail tendons incubated for 19 days in 0.1 M ribose, glucose-6-phosphate, and glucose. Spectra were obtained against a blank of collagen digest from tendons incubated in phosphate-buffered saline without sugar.

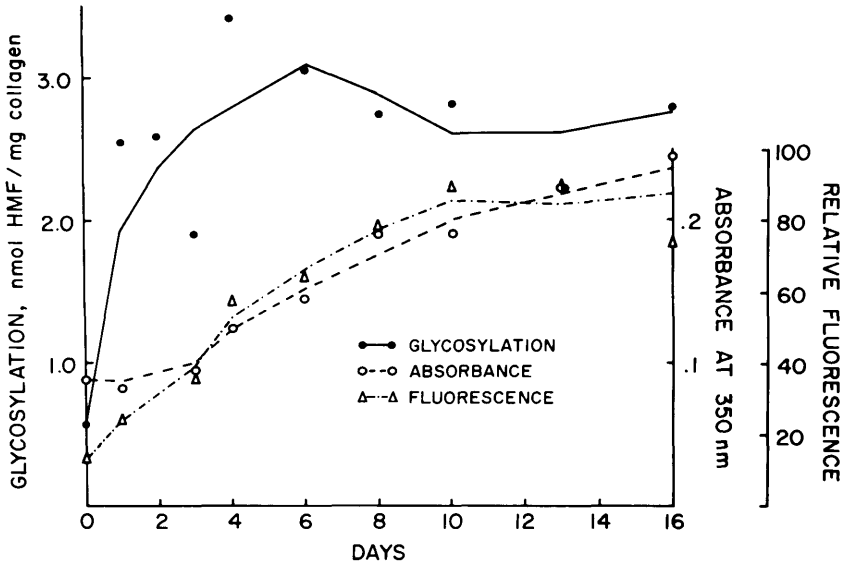

FIGURE 3. Time study of levels of nonenzymatic glycosylation (HMF) and amounts of fluorescent and pigmented compounds in digests of rat tail tendon incubated in 0.1 M glucose-6-phosphate at 37°C. Solutions were adjusted to contain 5 mg of collagen digest and 1 mg of enzyme. Absorbance at 350 nm was determined. Solutions were diluted 1:5 for fluorescence measurements. Relative fluorescence at 440 nm upon excitation at 370 nm was determined. Curves are drawn by the moving average method.

of aorta and tendon.[31] The glycosylated product, however, represents a noncrosslinked intermediate; it would be useful to determine if more definitive correlations of extent of lesions such as atherosclerotic plaques occurred with browning of collagen at sites of the lesions.

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