

Age-related Normalization of the Browning Rate of Collagen in Diabetic Subjects without Retinopathy

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Abstract

The age-related changes in collagen-linked fluorescence (browning) were investigated in skin from subjects with long-standing type I diabetes. Overall browning rates were 2.4 times higher in diabetics than in controls ($P < 0.02$) and slope intercept accurately reflected the mean age of onset of diabetes (11.6 vs. 11.2 yr), suggesting that the browning process has the attributes of a biological clock. Browning rates were not different in controls and diabetics without retinopathy ($P > 0.05$) but were 2.4 ($P < 0.05$) and 2.7 ($P < 0.01$) times increased in the presence of background and proliferative retinopathy, respectively. Compared with subjects with retinopathy, individual browning rates since onset of diabetes decreased with advancing age in subjects free of retinopathy ($P < 0.001$). Extrapolation revealed that they would become identical to that of nondiabetic subjects by the age of 66.4 yr.

These results suggest the presence of a mechanism that controls the browning rate of collagen in diabetics who do not develop retinopathy.

Introduction

Proteins that are exposed to glucose slowly undergo nonenzymatic browning. Yellow adducts and cross-links are formed that exhibit characteristic fluorescence at 440 nm upon excitation at 370 nm (1). This process is referred to as the Maillard reaction (2) and is a consequence of the nonenzymatic glycosylation reaction that is increased in diabetes. In a preliminary study, we found elevated amounts of Maillard-like fluorescence in dural collagen from three subjects with type I diabetes (1). Presumably, these fluorescent products accumulated slowly over time, thus reflecting cumulative glycemia over a period of years.

We have recently investigated the relationship between skin collagen-linked fluorescence and the severity of complications in 41 subjects with long-standing type I diabetes (3). Overall age-adjusted fluorescence was found to increase with the severity of retinopathy, nephropathy, and arterial and joint stiffness. Univariate analysis revealed that the correlation was significant for retinopathy, arterial, and joint stiffness as well as for blood pres-

sure and the sum of all complications. Multivariate analysis indicated age and presence of retinopathy as the most significant variables of collagen-linked fluorescence. Assuming that fluorescence was derived from glucose, we concluded that, in subjects with severe complications, cumulative glycemia had been higher over the years than it had been in those subjects without complications. This conclusion was based on the assumption that collagen browning rate was influenced by glycemia only. If, however, browning rates were to differ among individuals, it could be possible that the extent of collagen browning is not only related to metabolic level, but that in diabetic subjects who do not develop complications a mechanism is present that either prevents excessive browning or removes browning products more efficiently than it does in those with complications.

To obtain insight into this question, we have investigated the relationship between overall and individual browning rates of skin collagen and severity of retinopathy using the raw data from the study described above (3).

Methods

These have been fully described in reference 3 and are summarized here.

Subjects. 22 female and 19 male type I diabetics with a mean age of 36.4 yr (range, 29–52 yr) were enrolled. Mean diabetes duration was 25.1 yr (range, 20–40 yr) and mean age of onset was 11.2 yr. Presence of type I diabetes was based on the criteria of the National Diabetes Data Group (4). Mean insulin requirement was 45 U/d (range, 20–80 U/d). Age-matched nondiabetics (11 females and 14 males) served as controls.

Retinopathy was assessed using funduscopy with dilated pupils and graded 0 (no fundus abnormality, $n = 11$); 1 (background retinopathy, $n = 16$); and 2 (proliferative retinopathy, $n = 14$). Mean age \pm standard deviation was 35.9 ± 4.9 , 36.4 ± 4.8 , and 36.8 ± 5.9 yr, mean duration was 24.1 ± 6.0 , 24.6 ± 3.6 , and 26.0 ± 5.2 yr, and mean level of glycohemoglobin (Glycogel B; Pierce Chemical Co., Rockford, IL) was 15.7 ± 1.6 , 14.2 ± 3.2 , and $15.7 \pm 2.6\%$, respectively. These differences were not statistically significant. Informed consent was obtained from all participants.

Skin biopsy technique. Skin-punch biopsies (4 mm) were obtained under local anesthesia from the upper left buttock. Specimens were soaked for 60 min in saline, rinsed three times, and stored at -70°C under nitrogen until the end of the study. All analyses were done using identical batches of buffers and chemicals.

Solubilization of collagen. Epidermis and fat were removed from the frozen specimen with a razor blade. The remaining tissue was minced, suspended in 10 ml of deaerated cold phosphate-buffered saline (pH 7.4), and homogenized for 60 s with a Polytron homogenizer. The suspension was transferred into borosilicate tubes with Teflon-lined caps. Centrifugation was done at 650 g for 30 min at 4°C . The supernatant was removed and the pellet was washed three times with deaerated deionized water.

Lipids were extracted from the pellet with 5.0 ml of $\text{CHCl}_3/\text{MeOH}$ (2:1) by mild shaking overnight. 2 ml of methanol and 0.5 ml of water were then added to rehydrate the insoluble pellet. After centrifugation,

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the pellet was sequentially washed with methanol, 0.02 M Hepes buffer, pH 7.5, containing 0.1 M CaCl_2 (buffer H) and stored overnight at 4°C in buffer H. The pellet was digested for 24 h at 37°C in 1.0 ml of buffer H containing 280 U of type VII collagenase (Sigma Chemical Co., St. Louis, MO) and 2 μl each of chloroform and toluene to prevent bacterial growth. A residual pellet containing, on the average, <2% of total collagen was removed by centrifugation. The clear supernatant was used for assays of fluorescence and hydroxyproline content.

Collagen-linked fluorescence assay. Distilled water (1 ml) was added to the supernatant and fluorescence was measured against water at 440 nm, with excitation at 370 nm, as previously described (3). All values were corrected for fluorescence of the collagenase blank, which accounted, on the average, for <10% of control values. Fluorescence data were expressed in arbitrary units per milligram of collagen. Coefficient of variation was 11% for collagen-linked fluorescence in normal subjects.

Collagen content assay. Collagen content was assayed as described earlier (3). 50 ml of digest were hydrolyzed in 1.0 ml of 6 N HCl for 24 h at 110°C. 4-Hydroxyproline was assayed in the dry residue. Quantitative data are expressed per milligrams of collagen, assuming a 14% content of hydroxyproline (5).

Calculation of individual browning rates. Individual browning rates were calculated with the assumption that the diabetic rate departed from the nondiabetic control rate at the time of diabetes onset. Individual browning rate ($\text{IBR} = (\text{Ydb} - \text{Yc})/(\text{Xdb} - \text{Xc})$) whereby Ydb is the fluorescence value of diabetic subjects, Xdb is the age of diabetic subjects, and Yc is equal to $\text{AXc} + \text{B}$. A and B are the slope and y-intercept of the linear equation for nondiabetic controls (Fig. 1). Xc is the age at onset of diabetes, Xdb - Xc is the duration of diabetes, and $\text{IBR} = [\text{Ydb} - (\text{AXc} + \text{B})]/(\text{Xdb} - \text{Xc})$.

This formula was programmed into a computer. Individual browning rates were calculated and correlated with age of subjects (Fig. 3 and Table I).

Statistical analysis. Formulas for calculation of linear equations, regression coefficients, correlation coefficients, and multiple comparison (Tukey's method) were obtained from Zar (6) and programmed into a computer.

Results

The relationship between collagen-linked fluorescence and age of diabetic and control subjects is shown in Fig. 1. The accu-

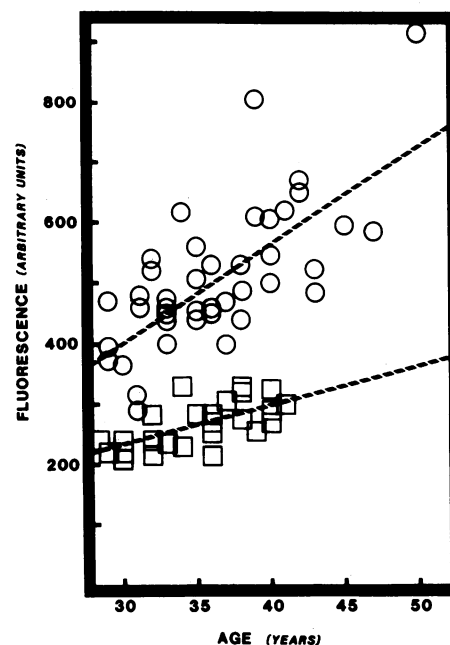


Figure 1. Relative fluorescence at 440 nm (excitation at 370 nm) per milligram of collagen in diabetics (\circ) ($y = 16.0x - 73.4$; $r = 0.77$; $P < 0.001$) and in controls (\square) ($y = 6.6x + 35.6$; $r = 0.65$; $P < 0.001$).

mulation rate of fluorescence (browning rate) was 2.4 times higher in diabetic than in control subjects ($P < 0.02$). The age at which both lines intercept was found to be 11.6 yr, which is very close to the mean age of diabetes onset of all subjects (11.2 yr). The relation between severity of retinopathy and browning rate as a function of age is shown in Fig. 2. Overall browning rate of the group without retinopathy (group 0) was not significantly different from that of controls ($P > 0.05$). In contrast, this rate was 2.4 ($P < 0.05$) and 2.7 ($P < 0.01$) times higher in the group with grades 1 and 2 retinopathy, respectively, than in the control group.

The observation that the overall fluorescence accumulation rate in subjects without retinopathy is not different from that of controls could either be an artefact due to similar duration of diabetes or could be due to the presence of a mechanism that prevents accumulation of browning products. To investigate these possibilities, we calculated individual browning rates, assuming that these departed sharply from the rate of nondiabetic controls at the onset of diabetes and that they were constant since that time. These assumptions are warranted by the observation that the regression lines in Fig. 1 intercept at the mean age of onset of diabetes. If the parallel slopes of grade 0 and control subjects (Fig. 2) were due to identical diabetes duration, one should find no change in individual browning rates with advancing age (slope = 0). If, on the contrary, the parallelism of the slopes in Fig. 2 was due to a mechanism preventing further browning, one would expect a decrease in individual browning rates with advancing age.

First, we found that the mean of individual browning rates increased with severity of retinopathy from 14.2 to 17.6 U of fluorescence per milligram collagen per year (Table I), confirming that not only the overall browning rates but also the individual browning rates are increased in diabetic retinopathy. The degree of significance is somewhat weak ($P < 0.05$) but is expected to be age-dependent, as suggested below.

Table I. Age-related Changes of Browning Rates of Collagen in Subjects with Varying Grades of Retinopathy

	Grade 0 (n = 11)	Grade 1 (n = 16)	Grade 2 (n = 14)
*Age-related browning rate (fluorescence in AU/yr)	8.8	16.2	18.1
‡Individual browning rate (fluorescence in AU/yr)	14.2 ± 1.78	15.8 ± 4.32	17.6 ± 3.09
§Age-related change in individual browning rate (fluorescence in AU/yr ²)	-0.25	0.27	0.14

AU, arbitrary units per milligram of collagen.

* Slope for grade 0 is not significantly different from that of control. Slopes for grades 1 and 2 are significantly different from the control slope with P values <0.05 and 0.01, respectively.

‡ Mean \pm SD of individual browning rates since onset of diabetes are significantly higher in grade 2 than in grade 0 retinopathy ($P < 0.05$). The mean of the grade 1 group is not significantly different from that of grades 0 and 2.

§ Slopes for grade 0 are significantly different from those of grades 1 and 2 ($P < 0.001$) but those for grades 1 and 2 are not significantly different from each other.

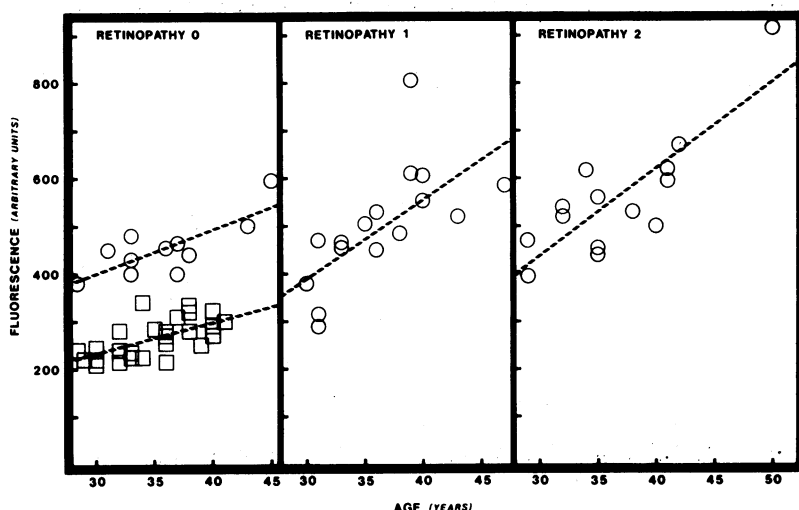


Figure 2. Age-related accumulation of collagen-linked fluorescence in diabetics (○) with grade 0 ($y = 8.76x + 138.8$; $r = 0.74$; $P < 0.01$), with grade 1 ($y = 16.25x - 89.6$; $r = 0.66$; $P < 0.01$), and with grade 2 retinopathy ($y = 18.1x - 106$; $r = 0.86$; $P < 0.01$) and in nondiabetic controls (□) shown again here for comparison purposes.

Second, when individual browning rates were plotted as a function of age, they were found to decrease linearly and significantly ($r = -0.68$, $P < 0.02$) with advancing age in subjects without retinopathy (Fig. 3). Compared with subjects with retinopathy, the decrease was highly significant ($P < 0.001$) (Table I). Borderline or no significant correlation with age was found in subjects with grades 1 and 2 retinopathy. Using the equation $y = -0.25x + 23.2$, we could calculate that, in subjects without retinopathy, individual browning rates would become identical to those of controls by the age of 66.4 yr.

Discussion

The rate analysis approach that we used to investigate collagen browning in type I diabetes revealed a number of surprising observations. First, the line intercept of control and diabetic browning rates in Fig. 1 predicts with a high degree of accuracy the mean age of onset of diabetes, suggesting that the process that modifies diabetic collagen occurs at constant rate and thus has the properties of a biological clock. It also suggests that diabetic skin collagen must have an extremely slow turnover rate. Second, when separated according to severity of diabetic retinopathy, the overall browning rate of subjects free of retinopathy is identical to that of controls (Fig. 2). Third, this phenomenon is found to be due to a significant decrease in individual browning rates with advancing age of the subject (Fig. 3), such that by the

projected age of 66.4 yr these rates reach normality. These latter observations strongly suggest that the reported association between collagen browning and severity of diabetic retinopathy (3) is not due only to differences in cumulative glycemia. If that had been the case, one would have expected the overall browning rate in the grade 0 retinopathy group to be significantly different from that of controls since none of these subjects were euglycemic. In fact, glycohemoglobin data and data on nonenzymatic glycosylation of collagen (7) were not significantly different among various retinopathy groups. Thus, one has to postulate that in subjects resistant to diabetic retinopathy, a mechanism is present that either prevents excessive browning of collagen or is efficient in removing modified collagen. Accordingly, this mechanism would be absent or less efficient in diabetics with background and proliferative retinopathy.

Browning of collagen can theoretically result from a number of processes that can be of an enzymatic (8) or of a nonenzymatic nature (9, 10). Our assumption that it involves browning of Amadori products during the late stages of the Maillard reaction is based on circumstantial evidence; other mechanisms cannot be excluded at the present time. In vitro, the glucose-mediated browning rate is strongly dependent on concentration of the sugar (11). Other factors, like type of sugar, pH, and the presence of oxygen and metals, however, have been found to influence the browning rate (12). Recent work by Baynes and his associates (13) indicates that Amadori products of lysyl residues are de-

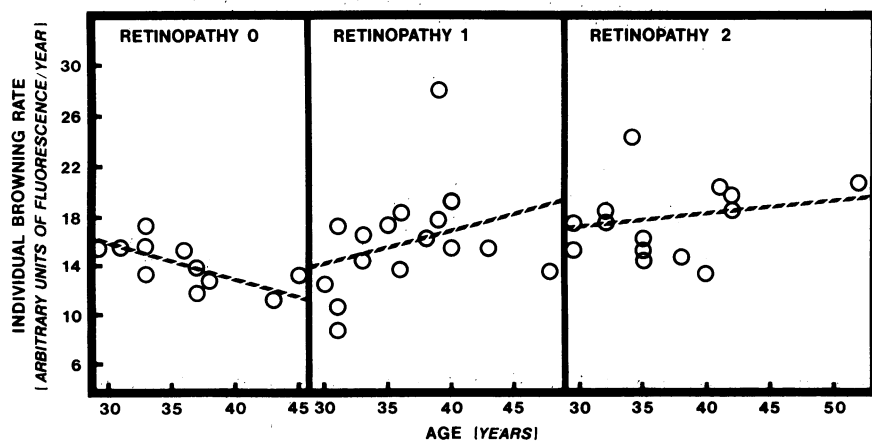


Figure 3. Individual browning rates of collagen as a function of age in diabetic subjects with varying severity of retinopathy. Grade 0 ($y = -0.25x + 23.2$; $r = -0.68$; $P < 0.02$), grade 1 ($y = 0.27x + 6.08$; $r = 0.31$; $P > 0.05$), and grade 2 ($y = 0.14x + 12.3$; $r = 0.29$; $P > 0.05$).

graded into ϵ -carboxymethyllysine in the presence of O_2 and Fe^{2+} . The carboxymethyl group blocks the ϵ -amino group of lysine and prevents it from undergoing browning. Increased formation of this adduct in subjects without retinopathy would effectively decrease Maillard-mediated browning and cross-linking of collagen. Since anoxia has been implicated in diabetic retinopathy (14), it is possible that subjects without retinopathy would have better tissue oxygenation and lower browning rates of Amadori products due to increased formation of ϵ -carboxymethyllysine. The fact that this amino acid has been detected in human urine (15) supports this possibility.

An alternative regulatory mechanism that should be considered is one that would involve accelerated removal of browning products or browned collagen. Vlassara and colleagues have shown that proteins modified by advanced glycosylation endproducts (browning products) in vitro or in vivo (16), as well as proteins modified by the Maillard cross-link furoyl furanyl imidazole were preferentially taken up by macrophages (17). These experiments suggest that macrophages have a specific receptor for advanced glycosylation endproducts and that they could act as bioregulators of the Maillard-mediated aging process. Decreased removal of browned collagen in subjects with retinopathy could be due to a down-regulation of these receptors.

Finally, the recent discovery of analogues of pyridinoline in human skin suggests the possibility of a browning mechanism based on lysyl oxidase-mediated modification of lysyl residues (18). Increased lysyl oxidase activity (19), as well as lysyl oxidase-mediated cross-linking of collagen, have been documented in experimental diabetes (20). It could well be that, in diabetic subjects without retinopathy, formation of pyridinoline-like compounds occurs only during adolescence, as in nondiabetics (21), but increases throughout life in those subjects who develop diabetic complications. This would explain why browning rates were highest at a younger age in subjects without retinopathy.

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