Involvement of Hydrogen Peroxide in Collagen Cross-linking by High Glucose in Vitro and in Vivo

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The Maillard reaction has been implicated in cross-linking and fluorescence formation of collagen exposed to high glucose in vitro. However, several pharmacologic agents, whose action seems unrelated to pathways of nonenzymatic glycation, have been demonstrated to prevent cross-linking in diabetes. To clarify this discrepancy, kinetic changes in glycation, glycoxidation (carboxymethyllysine, CML), and cross-linking (measured as tendon breaking time, TBT) were evaluated in rat tail tendons incubated in 5 and 30 mM glucose in vitro and in tendons implanted in vivo into diabetic rat peritoneal cavity. In vitro, rates were found to be both O2- and glucose-dependent. Tendon preglycation and presence of added 2 mM glycosylamine and Amadori compounds (Amadori product of glucose and propylamine) catalyzed these changes in a primarily O2-dependent manner. In the presence of Amadori compounds, kinetic changes were dramatically increased and were preventable by addition of catalase to the medium. Tendons implanted into diabetic rat peritoneum became more rapidly glycoxidized and cross-linked when implanted at day 30 from diabetes onset (high tissue glycation) compared to day 3 (low tissue glycation) in spite of similar glycation kinetics, suggesting a mechanistic disassociation between glycation, glycoxidation, and cross-linking in diabetes. Indeed, intraperitoneal injection of catalase and other antioxidants dramatically suppressed cross-linking, fluorescence formation, and, to some extent, glycoxidation, without affecting glycation. This study confirms the role of oxidative stress in protein cross-linking by the Maillard reaction in vitro and provides the first evidence for a role of H2O2 in cross-linking in diabetes. Whereas Amadori products are a potent source of H2O2 formation in vitro, their precise contribution to H2O2 generation and the actual role of Maillard reaction products in collagen cross-linking in diabetes requires further investigation.

Considerable efforts have been devoted in recent years toward understanding of the pathogenetic mechanisms leading to collagen insolubilization in diabetes and aging (1–3). The rationale for this research lies in the hope that understanding the biochemical basis of stochastic mechanisms of damage to collagen will help elucidate their role in diabetic complications and aging. One key proposition is that protein cross-links are generated in the advanced Maillard reaction (4, 5). According to the original concept proposed by Hodge (6), the central molecule responsible for the advanced Maillard reaction is the Amadori product which, upon dehydration and rearrangement, forms highly reactive deoxyosones—potent precursors of protein cross-links. This concept was challenged by Namiki (7) who found that sugar fragmentation can also occur at the stage of the glycosylamine, or Schiff base, i.e. prior to the Amadori rearrangement. Glycoxal is then formed which can serve as a protein cross-link (8–10). More recently, Wolff et al. (11) obtained evidence from in vitro experimentation that metal-catalyzed glucose autoxidation may be more important than glycation for generation of fluorescent protein adducts and cross-links. These observations raise considerable questions concerning the actual role of the Maillard reaction in the modification of proteins and the generation of diabetic complications (12).

The notion that oxidation reactions may be playing an important role in collagen cross-linking and fluorescence formation by high glucose during the Maillard reaction originates from a series of elegant in vitro studies by Baynes and colleagues (2, 13). These investigators showed that removal of oxygen and the presence of metal chelators or free radical scavengers could uncouple collagen cross-linking and glycoxidation from collagen glycation (13). Similar experiments by Chace (14) showed that collagen insolubilization in the presence of glucose was dependent on the formation of reactive oxygen species. On the other hand, treatment of diabetic rats with nonsteroidal anti-inflammatory agents (aspirin, salicylic acid, indomethacin, and naproxen) (15, 16), aldose reductase inhibitors (sorbinil, rutin (17, 18), antioxidants (α-tocopherolamine, vitamin E (19, 20)), or scavengers of dicarbonyl compounds (aminoguanidine and l-arginine (18, 21, 22)), all prevented increases in either collagen-linked fluorescence, thermal stability, or both. In the case of aldose reductase inhibitors, contradictory results were obtained (23, 24).

Thus, although current in vitro data suggest that an oxidative mechanism coupled to the generation of reactive ketoaldehydes might be responsible for collagen cross-linking by high glucose (13), the great diversity in the pharmacological spectrum of action of the drugs above contributes toward blurring the mechanistic relationship between glycation and cross-link formation. Similarly, work by Brennan (25, 26) directly questioned the relationship between glycation and collagen cross-linking in diabetes. In addition, observations by Wolff et al. (12) suggest that glucose autoxidation rather than glycation is more important for protein modification, findings contradicted by the more recent data of Hunt et al. (27).

This study was initiated with the goal of clarifying the in
vitro and in vivo role of a high glucose environment on the increase in glycoxidation (carboxymethyllysine) and thermal stability of collagen as measured by the tail tendon breaking time assay (TBT). The TBT assay has proven value as a highly sensitive marker of collagen cross-linking both in diabetes and gerontological research. In contrast to previous studies in which supraphysiological concentrations of glucose and phosphate buffer were used (13), we designed incubation conditions which would mimic as closely as possible both eu-glycemic and diabetic milieus. In the second phase of the study, tail tendons were “incubated” in the peritoneal cavity of hyper-glycemic rats. The results of this combined approach lead to implication of hydroxide peroxide in collagen cross-linking in vivo and by the Maillard reaction in vitro.

**EXPERIMENTAL PROCEDURES**

**Reagents**

Reagents of the highest quality available were obtained from Sigma, unless indicated otherwise. 1-Deoxyfructosyl propylamine (Amadori product) and N-glucosyl propylamine (Schiff base adduct) were prepared according to the method of Michaelis and Hagemann (28). Briefly, 28 g (0.16 mol) of anhydrous glucose was dissolved in 28 ml (0.34 mol) of propylamine at 70°C. After almost complete dissolution, 100 ml of 2-propanol was added, and the solution was poured immediately into 800 ml of diethyl ether. The colorless precipitate (N-glucosyl propylamine) was separated and washed extensively with diethylether. Following complete removal of the ether, the material was dissolved in boiling 1,4-dioxane, and, under stirring, 20 g (0.16 mol) of oxalic acid diethylester was added. The resulting dioxane/methanol (1:1) to yield 16 g (37%) of colorless crystals of 1-deoxyfructosyl propylamine-oxalate. Analytical data complied with the literature.

One hundred female Sprague-Dawley rats (2 months old), weighing between 200 and 220 g, were used as a source of tail tendons. The animals were killed by CO2 narcosis. The tails were stored in intact form at ~80°C until use. Tendons were prepared as described previously (29).

**Incubation Conditions**

Intact tail tendons (about 50 per tube) were incubated in 20 ml of deaerated 0.01 M phosphate-buffered saline (PBS) (pH 7.4) in sterile tubes at 37°C with or without 5 and 30 mM glucose under aerobic or antioxidant conditions. Antioxidant conditions were established by adding 1 mM of diethylene triaminepentaacetic acid to chelate traces of transition metal ions which act as catalyst of oxidation reactions (13). Oxygen was removed by flushing of the reaction mixture with nitrogen for 5 min and sealing under a stream of nitrogen. Toluene and chloroform (each 2 μl/ml) were added to prevent microbial growth. For selected experiments, tendons were first preincubated under anaerobic conditions in 100 mM glucose for 10 days to induce glycation. The peglycated tendons were then washed free of excess glucose and incubated under standard conditions. In other experiments, 2 mM concentrations of N-glucosylpropylamine or Amadori product (1-deoxyfructosyl propylamine) were added to the standard incubation conditions. At days 3, 7, 14, and 28, five tendons each were removed, washed in deaerated PBS, and stored at ~70°C until assay. After each removal, tubes were flushed again with nitrogen and sealed prior to further incubation. Each tendon was divided into halves, whereby the proximal end was used for tendon breaking time measurement and the distal end for biochemical assays.

**Intraperitoneal Implantation of Tail Tendons**

All animal experiments were approved by the Institutional Animal Care and Utilization Committee (IAUC). Seventy-five female Sprague-Dawley rats, 2 months old, weighing 200 to 220 g, were divided into two groups consisting of 15 normal rats and 25 rats in whom diabetes was induced by streptozotocin injection into the tail vein (65 mg of streptozotocin/kg body weight in citrate buffer (pH 4.5)). Diabetes was confirmed by Diastix (urinalysis) from Miles Co. (Tarrytown, NY), and glucose levels were quantified with a One Touch Strip from Lifescan (Milpitas, CA). For surgery in the diabetic rat, anesthesia consisted of a mixture made of 3 parts of ketamine (100 mg/ml), 2 parts of xylazine (20 mg/ml), and two parts of normal saline. One ml/kg of this mixture injected intraperitoneally induced sleep for a period of up to 45 min. Nondiabetic rats received a mixture of 0.1 ml of xylazine (20 mg/ml) with 0.9 ml of ketamine (100 mg/ml) at a dose of 0.12–0.15 mg/kg intra-peritoneally. After removal of hair and dissection, a 0.5-cm long sagittal incision was performed in the abdominal wall and a bundle of 6 tendons which were kept in culture medium containing penicillin/streptomycin (100 units/ml and 100 μg/ml, respectively) were implanted into the peritoneal cavity. The cavity was closed with a single 5-0 suture. Experiment 1—12 diabetic rats (Group I) were implanted 3 days after induction of diabetes. Thereafter, 5–7 rats each were sacrificed on days 7, 14, and 28 after implantation, i.e. 10, 17, and 31 days after diabetes onset. A second set of diabetic rats (Group 2) was implanted after 31 days of diabetes, and, similarly, 5-7 rats each were sacrificed on days 7, 14, and 28 after implantation, i.e. 38, 45, and 59 days after onset. In all groups, glycemia and glycohemoglobin levels were determined at appropriate time points as described in Tables II and III.

Experiment 2—Diabetic rats were divided into seven groups following implantation. They were treated daily with the following regimen: Group I was without treatment (control), group II received sterile filtered α-penicillamine hydrochloride intraperitoneally (200 mg/kg body weight dissolved in 0.9% saline, pH 6.5), group III received deferoxamine subcutaneously (85 mg/kg body weight in saline), group IV was fed probucol (1% w/w in food), group V received catalase injected intraperitoneally (65,000 units 0.5 ml of saline equivalent to 0.1 mg of protein daily), group VI received 0.1 mg of bovine serum albumin (BSA) in 0.5 ml of saline intraperitoneally in amounts identical with catalase. Group VII received aminoguanidine hydrochloride in drinking water (1 g/l). After 20 days of treatment, all rats were killed by cardiac puncture. For all experiments, tendons were washed twice with deaerated PBS and stored in deaerated PBS at ~80°C until analyzed.

**Analytical Procedures**

**Tendon Breaking Time Assay—**Tendon collagen cross-linking was assayed by the sensitive method of breaking time measurement as previously described (29). Briefly, tendons were attached on one end with a 4.0 surgical nylon string to an electric switch connected to a timer. A 2.75 ± 0.2 g lead weight was attached to the lower end of the tendon. Tendon and weight were immersed in fresh 7 M urea solution (pH 7.5) as described previously (29) at a rate of 0.1 ml of 7 M urea solution per minute. Temperature of the urea solution was kept constant at 40°C ± 0.5 by a surrounding bath. Time until tendon rupture was measured.

Collagen-linked Fluorescence—Collagen-linked fluorescence was measured as described previously by us (29) with some modifications. Tendons (10 mg wet weight) were washed with PBS, blotted on filter paper, minced, and digested by 2% pepsin (w/w) in 0.5 N acetic acid for 17 h at 37°C in a rotating extraction machine (Glasco-Cd, Newtont, CT). After centrifuging for 30 min at 15,000 g, the remaining pellet was found to contain, on the average, less than 2% of total collagen. The clear supernatant containing digested collagen was used for assays of fluorescence, hydroxyproline, pentosidine, carboxymethyllysine, and fuoresine.

For fluorescence measurement, 90 μl of the supernatant were added to 2 ml of deionized water, and fluorescence was measured at 370 nm excitation and 440 nm emission. All fluorescence values were corrected for fluorescence of the pepsin blank and expressed per mg of collagen.

**Acid Hydrolysis—**Approximately 2 mg of pepsin-digested collagen were hydrolyzed with a Savant centrifugal evaporator and taken up in 1 ml of 6N HCl. Samples were flushed with nitrogen, incubated at 110°C for 18 h, dried under vacuum, and reconstituted in 1 ml of HPLC solvent A (1000 ml of water + 1.3 ml of heptfluoroctylic acid).

**Hydroxyproline—**Hydroxyproline was assayed in the acid hydrolysate as described previously (29). Collagen content was calculated assuming 13% content of hydroxyproline.

**Carboxymethyllysine Assay (CML)—**Carboxymethyllysine was assayed using o-phthalaldehyde (OPA) method and post-column derivatization as recently described by us (8). System 1 consisted of water (elucent A) and 70% methanol in water (elucent B), both with 0.01 M heptfluoroctylic acid (Alfradich), reverse phase C18-column (0.4×25 cm, 5 μm, Ydac 218TP54, The Separations Groups, Hesperia, CA), flow rate 1 ml/min, gradient 0–2% B for 10 min, then in 5 min to 100% B, System 2, column and flow as for system 1, with 5% propanol (elucent A) and 60% propanol in water (elucent B), both with 3 g of sodium sulfate.

1 The abbreviations used are: TBT, tendon breaking time; CML, N-(carboxymethyl)lysine; HPLC, high performance liquid chromatography; Amadori product, product of glucose and propylamine; BSA, bovine serum albumin; glycoxidation, H-glucosyl propylamine; PBS, phosphate-buffered saline.
(SDS) (Fluka Buchs, Switzerland) and 1 g of monobasic sodium phosphate monohydrate adjusted to pH 2.8 with phosphoric acid. The gradient was 15% B to 22% B in 30 min, 22% to 40% B in 20 min, and 40% B to 100% B in 5 min.

Furosine Assay—Quantification of furosine was performed by HPLC with an on-line absorbance detection system set at 280 nm wavelength with high sensitivity setup at 0.01 absorbance unit at full scale (model 484, Waters Co., Milford, MA), using a reverse phase C8 column with solvent A containing 0.04% (v/v) acetic acid in water and solvent B containing 0.27% (w/v) potassium chloride in solvent A as described before by Resmini (30).

Determination of Glycated Hemoglobin—Heparinized blood collected by cardiac puncture was centrifuged with a Beckman Microfuge to separate erythrocytes. Fifty microliters of erythrocyte saline mixtures (1:1 v/v) were lysed with 500 μl of preparative reagent for quantification by boronate affinity chromatography with the GlycoTest II from Pierce (Catalog No. 45100). The manufacturer’s instructions were followed.

Statistical Analysis—Means and standard deviations were calculated for each timepoint of the tendon breaking time experiment using 7 tendons. Analysis of variance using Newman Keuls multiple comparison test was performed to indicate differences between treatment groups. p values less than 0.05 were considered statistically significant.

Furosine and carboxymethyllysine values for in vitro experiments represent single determinations in pooled specimens of tendons. Data obtained with intraperitoneally implanted tendons are expressed as means and standard deviations utilizing 6 tendons per rat.

RESULTS

Base line or “standard” conditions were first established by incubating intact tail tendons for up to 28 days with 5 and 30 mM glucose in phosphate-buffered saline. As expected based on the work of Fu et al. (13), glycation (furosine) and carboxymethyllysine (CML) increased faster in high versus low glucose concentrations, and CML formation was suppressed by anaerobic conditions (Fig. 1). Surprisingly, tendon breaking time (TBT) increase was more strongly influenced by the presence of oxygen rather than by glucose concentration. Tendon breaking time in tendons incubated without glucose increased at a slower rate than in 5 mM glucose and was virtually absent under anaerobic conditions (not shown). The lower glycation rate under N2 is unexpected. It was observed in most of experiments presented below and may relate to differences in experimental conditions, whereby Fu et al. (13) used high salt for prior extraction of soluble proteins, as well as high concentrations of glucose and phosphate, both of which catalyze glycation (31).

In order to investigate the impact of pre-existing tendon-linked Amadori products on cross-linking and glycoxidation, identical incubations were carried out with tendons that were first preglycated for 10 days under anaerobic conditions in order to minimize cross-linking and glycoxidation. Under such conditions, base line levels of all parameters were higher at time 0 and further increased upon incubation (Fig. 2). Again, the presence of 30 mM glucose concentration and aerobic conditions further increased CML and TBT values. Compared to the base line conditions (Fig. 1B), however, net increase in CML levels reached much higher levels at both 5 and 30 mM glucose concentrations when oxygen was present (Fig. 2B). Similarly, whereas TBT increased by less than 5 min between day 3 and day 28 under standard conditions in O2 (Fig. 1C), it increased by more than 10 min when tendons were preglycated (Fig. 2C). Interestingly, although the glycation rate (furosine) kept increasing in 30 mM, but not in 5 mM glucose (Fig. 2A), tendon breaking time increased independently of the subsequent changes in glycation, and essentially independently of the ambient glucose concentration. In comparison with the standard conditions, it thus appears that the changes in TBT are accelerated by preglycation and presence of aerobic conditions. To confirm this observation, TBT and CML changes were also determined in preglycated tendons that were incubated without glucose (Fig. 2C). The results revealed that CML and
TBT increases were very similar to those of tendons incubated in 5 or 30 mM glucose, suggesting that preformed Amadori products play an important role in TBT increase. The overall pattern of changes in TBT shared more similarities with those of CML formation than glycation (Fig. 2B), although some dissociation between the two are apparent in that CML did not increase in tendons incubated under anaerobic conditions in 5 mM glucose.

To further narrow down the mechanism of collagen cross-linking by the Maillard reaction, tendons were incubated under base line conditions in the presence of added N-glycosyl propylamine, a Schiff base precursor, or 1-deoxyfructosyl propylamine, i.e. its Amadori rearrangement product. Two millimolar concentrations were selected to simulate the plasma fructosamine levels in diabetes (56). The presence of Schiff base precursor had no major impact on glycation rate and TBT increase, except for an enhancement of CML levels (not shown). In contrast, however, the presence of 2 mM concentrations of Amadori product free in solution markedly catalyzed CML formation and TBT increase (Fig. 3, B and C) compared with standard conditions (Fig. 1, B and C). Moreover, TBT and CML increases were independent of ambient glucose concentration and strongly dependent on the presence of aerobic conditions, whereas the pattern of tendon glycation remained unaltered compared to the base line conditions (Fig. 3A).

The data so far indicate that the most important factors in catalyzing glucose-mediated increase in TBT are tendon pre-glycation and the presence of 2 mM Amadori products. In the case of CML, both the presence of glycosylamine and Amadori products were major contributors to CML formation, most likely by a mechanism involving glyoxal formation (8, 9). Thus, this suggests that glucose autoxidation is of minor importance for collagen cross-linking when compared with physiological concentrations of reactants. At supraphysiological glucose concentrations, however, it is a potent generator of glyoxal which can mediate protein cross-linking (8, 9, 32).

As an approach toward in vivo testing of the relative relevance of glucose autoxidation versus Amadori product oxidation in TBT increase and glycooxidation in vivo, we have performed in vivo “incubation” experiments by implanting tail tendons into the peritoneal cavity of diabetic rats. Tendons were implanted into each of 5 animals per group 3 days after the onset of diabetes (when total body protein glycation is still low), or after 28 days of diabetes (when protein glycation had reached steady state levels) (Table I). All parameters of collagen modification were measured as described before.

As expected, tendon glycation increased progressively with time and was higher in the diabetic compared to the control rats (Fig. 4A). Most importantly, glycation kinetics were identical in both rat groups, reflecting thereby identical mean glycemia during the course of the experiment, regardless of whether tendons were implanted at diabetes onset or when diabetes was in a steady state. In contrast, both rates of CML formation and TBT increase in the animals in whom tendons were implanted after 28 days of diabetes (Fig. 4, B and C) (Table I). Statistical significance was reached only for TBT (p < 0.05). It is thus clear that glycemia alone is not the determinant of the increase in CML and TBT.

Comparison of the in vivo kinetics of modification of tendons implanted at 28 days with the in vitro data obtained with 30 mM glucose concentration in the presence of oxygen (Table II) shows that glycation rate measured in vivo is roughly half that of the in vitro experiments. In spite of lower glucose concentrations in vivo, CML levels resembled closely the in vitro pattern (Fig. 1B). These data suggest the presence of a catalytic process present in vivo but not in vitro. Evidence for such a catalytic process was more readily apparent in the TBT experiment in which the rate of increase was best approximated by the presence of 2 mM concentrations of added Amadori products (Fig. 3, inset).

The data in Table II reemphasize the notion that Amadori products appear to play an important role in tendon cross-linking, as reflected by the TBT increase, but at the same time reveal that no in vitro model can satisfactorily account for all in vivo changes. Reports by Kawakishi et al. (33) and Jiang et al. (34) suggest that hydrogen peroxide is generated during in vivo modification of collagen.
vitro glucose incubation or in the presence of Amadori products. Therefore, we incubated tendons with 2 mM Amadori product in the presence of catalase (Fig. 5). A highly effective diminution of TBT increase was noted with native but not with heat-inactivated catalase, thereby implicating \( \text{H}_2\text{O}_2 \) in the Amadori product-mediated increase in heat stability of the tendons. CML was not determined in this experiment because two groups have shown unequivocally that catalase suppresses CML formation (35, 36).

Although these data implicate \( \text{H}_2\text{O}_2 \) in cross-linking of collagen exposed to Amadori product in vitro, the role of \( \text{H}_2\text{O}_2 \) for collagen cross-linking in vivo remained to be established. Taking advantage of the in vivo incubation model consisting of intraperitoneally implanted tendons, 7 groups of 5 rats each were implanted with tendons for 20 days and treated with \( \text{D-} \text{penicillamine, deferoxamine, probucol, catalase, bovine serum albumin, and aminoguanidine as described under "Experimental Procedures."} \) Percent of glycated hemoglobin at day 20 was 12% on the average and not significantly different between groups (Table III). Reference values in nondiabetic rats are 3.5–4.5% (Table I). Mean furosine level of intraperitoneally implanted tendons was elevated and not significantly different between each group (Fig. 6A). In contrast, a more pronounced, although not significant, decrease in carboxymethyllysine was noted especially in animals receiving penicillamine, probucol, and catalase (Fig. 6B). That trend became statistically significant for TBT (Fig. 6C), the increase of which was completely prevented by \( \text{D-} \text{penicillamine} \) and not significantly different from normal in animals receiving probucol or catalase injections. Levels in the BSA protein control and deferoxamine groups were slightly but not significantly decreased. No data could be obtained in the aminoguanidine-treated rats because of a fibrotic reaction which made the tendons unretrievable from the peritoneal cavity.

The beneficial effects of penicillamine, probucol, and catalase on collagen modifications by diabetes were further confirmed through measurement of collagen-associated fluorescence at 440 nm upon excitation at 370 nm (Fig. 6D). All three agents decreased fluorophore formation, whereas only a minor and not significant decrease was observed with deferoxamine and BSA.

The question of whether the intraperitoneal cavity is a physiological milieu for implanted tendons or whether it artificially catalyzes glycation, CML formation, and TBT increase due to a localized oxidative stress (32, 35) was investigated by comparing these parameters with those of the rats’ own tail tendons in the first 28 days after onset of diabetes. Glycation and CML formation rates were decreased by 25% and 50% compared to the implanted tendons, suggesting indeed that a local oxidative stress might enhance glycoxidation (not shown). In contrast, however, TBT increase at each time point was rigorously identical in both the native and implanted tendon (not shown).

**Table II**

| Experimental conditions | Furosine pmol/mg collected/ day | Carboxymethyllysine pmol/mg collagen | Tendon breaking time min/day |
|-------------------------|-------------------------------|-------------------------------------|-----------------------------|
| Glucose only            | 15.2                          | 1.00                                | 0.24                        |
| Glucose, preglycated tendon | 15.0                          | 1.43                                | 0.44                        |
| Glucose + 2 mM glycosylamine | 16.2                          | 2.03                                | 0.32                        |
| Glucose + 2 mM Amadori product | 14.3                          | 3.20                                | 1.43*                       |
| Tendons implanted at diabetes onset | 7.8                          | 0.55                                | 0.98                        |
| Tendons implanted at day 30 | 7.8                          | 0.82                                | 1.32                        |

*This value was computed for the first 15 days of incubation (fig. 5). It compares well with the 28-day value in Fig. 6 (2.14 min/day).
thereby validating the model of tendon implantation for mechanistic studies of collagen cross-linking by high glucose in vivo.

**DISCUSSION**

The purpose of our study was to clarify the relationship between high glucose concentrations in vitro, glycation, glycoxidation, and tendon collagen cross-linking and to compare the results with similar modifications resulting from the diabetic state in a model consisting of tendons implanted into the diabetic animal. This approach was utilized in order to allow us direct testing of both the effects of glycation (low versus high) and antioxidant enzyme (catalase) on collagen cross-linking in vivo.

While this study was in progress, a study by Fu et al. (13) showed that high concentrations of glucose and phosphate led to tail tendon collagen cross-linking and glycoxidation which could be uncoupled from glycation by the use of chelating agents, antioxidants, or aminoguanidine. Similar results were obtained in the present study with 30 mM glucose concentrations. Whereas Fu et al. (13) concluded that most of the observed changes could be attributed to events occurring before the formation of Amadori products, we show that preglycation or the presence in solution of small quantities of Amadori products compared to glucose concentrations markedly catalyzed glycoxidation and cross-linking compared to standard conditions (Fig. 1). These modifications are H$_2$O$_2$-dependent and essentially independent of ambient glucose concentration.

This raises doubts that glucose autoxidation is a major factor in tendon breaking time increase, although it is certainly expected to contribute to CML formation and cross-linking in vivo via glyoxal formation as recently shown by us and Wells-Knecht et al. (32).

Although the experiments presented above and by others (12, 33, 35, 36) implicate Amadori products in the formation of H$_2$O$_2$ in vitro, several questions arise concerning the mechanism and source of H$_2$O$_2$ formation in vivo with relationship to the Maillard reaction. Current concepts linking H$_2$O$_2$ formation from reducing sugars involve metal-catalyzed oxidation of enediols (27, 33). Amadori products could form H$_2$O$_2$ via 2 pathways (Fig. 7A). One pathway is the 1,2-enolization pathway which leads to 3-deoxyglucosone formation under anaerobic conditions. In the presence of a suitable electron acceptor, however, enolization would occur to form H$_2$O$_2$ and glucosone (33, 37). The other pathway is the 2,3-enolization pathway which leads to 1-deoxyglucosone and the putative 1,4-dideoxyglucosone (38). Under oxidative conditions, however, the 2,3-
enediol is thought to generate $\text{H}_2\text{O}_2$ and carboxymethyllysine (33, 39). Both Fe$^{11}$ and Cu$^{1}$ can serve as electron acceptors. In vitro experiments by Fu et al. (13) showed that the chelators and free radical scavengers Tiron and D-penicillamine could completely prevent glycoxidation and cross-linking. In our experiments, however, the iron chelator deferoxamine had no effect on any of the collagen modifications. This finding excludes iron as a major participant in the reaction leading to cross-linking. In contrast, the potent effect of D-penicillamine in blocking all modifications except for a weak effect on glycation suggests that Cu$^{2+}$ may be involved. Indeed, Cu$^{2+}$ concentrations have been reported to be increased in diabetes (40), and D-penicillamine has been found to chelate Cu$^{2+}$ (41). On the other hand, Jiang et al. (34), while performing in vitro glycation experiments, found that albumin which chelates Cu$^{2+}$ (42) substantially blocked $\text{H}_2\text{O}_2$ formation. Since daily intraperitoneal injection of albumin had no effect in our experiments, it would appear unlikely that Cu$^{2+}$, at least in free form, would contribute significantly to $\text{H}_2\text{O}_2$ formation. There is, however, recent evidence which suggests that protein-bound Cu$^{1}$, e.g. in form of ceruloplasmin, can have oxidizing activity (43). Thus, a catalytic role of copper in collagen cross-linking in vivo cannot be excluded.

Another mechanism by which D-penicillamine could exert its beneficial effect is as an antioxidant through its SH group. In support of this possibility is the finding that propocrol, a disulfide-linked antioxidant, inhibited the collagen changes in vivo.

Assuming the increased levels of $\text{H}_2\text{O}_2$ are generated by the Amadori product, the next question is whether the sugar itself is involved in cross-linking. Clarification of that question is important, since the experiments above have shown that catalase inhibits cross-linking, implying thereby a possible direct effect of $\text{H}_2\text{O}_2$ on cross-linking. Indeed, we were able to reproduce the biphasic cross-linking pattern observed with Amadori product in solution (Fig. 3C) by incubating the tendons with 10 mM $\text{H}_2\text{O}_2$ in PBS (not shown). We were however unable to test the effects of aminoguanidine as a trapping agent of dicarbonyl compounds due to a fibrinotic reaction which prevented us from retrieving the tendons from the peritoneal cavity. However, there are both in vitro and in vivo data which show that aminoguanidine is a potent inhibitor of collagen cross-linking (20). To further clarify the role of the sugar versus that of $\text{H}_2\text{O}_2$, we incubated tendons for 24 h only with 10 mM $\text{H}_2\text{O}_2$ with or without 10 $\mu$M Cu$^{1}$ and compared the results with similar tendons incubated with 100 mM ribose in aerated phosphate buffer. Whereas immediate TBT increase was noticed with ribose, no change was observed in Cu$^{2+}/\text{H}_2\text{O}_2$ incubated specimens (not shown), thereby proving the necessity of involving a sugar in the early phase of collagen cross-linking as observed earlier by Chace et al. (14).

Thus, our results suggest that the Maillard reaction in combination with $\text{H}_2\text{O}_2$ formation is involved in collagen cross-linking. Furthermore, an overall relationship between cross-linking and CML formation is apparent, leading us to propose a common precursor for CML and cross-linking based on the Bayer-Villiger oxidation of the enedial Amadori product as shown in Fig. 7B. The cross-link is in essence a lysine dimer linked by a carboxymethylamide bridge. The amide nature of the cross-link would explain why the covalent incorporation of $\epsilon$-aminolysine into glycated protein was found to be labile to both acid and base hydrolysis, as well as nonreducible by borohydride (35). It should be noted, however, that a similar cross-link would form from the reaction of glyoxal with $\epsilon$-aminolysine (9).

In spite of its attractiveness, other cross-linking models need to be considered. $\text{H}_2\text{O}_2$ can originate from sources other than sugar oxidation and could catalyze collagen cross-linking, e.g. through lipid peroxidation (44) or direct formation of carbonyl groups on the protein as proposed by Stadtman (45). Lipid peroxidation, however, is unlikely to be involved since many studies have shown that it is not preventable by catalase (46, 47). Another possibility is based on Brennan's work (26) in which no evidence for the formation of new cross-links in tendons from diabetic rats could be found, suggesting that maturation of labile into stable cross-links may be occurring in diabetes. It may thus be that $\text{H}_2\text{O}_2$ acts by oxidizing pre-existing cross-links that are otherwise labile in the 7 M urea used in the TBT assay.

A number of sources other than Amadori products are conceivable for $\text{H}_2\text{O}_2$ in experimental diabetes. These potentially include decreased antioxidant defense such as low levels of ascorbic acid, vitamin E, uric acid, glutathione (48-51), and the antioxidant enzymes catalase and glutathione peroxidase (52). In addition, increased production or leakage of $\text{H}_2\text{O}_2$ by mitochondria, and increased activity of $\text{H}_2\text{O}_2$ generating enzymes such serum amine oxidase (52) and lysyl oxidase (53, 54) may account for the $\text{H}_2\text{O}_2$-mediated collagen cross-linking in diabetes. The latter possibilities are supported by studies showing that aminoguanidine is an inhibitor of the former enzyme (53), whereas D-penicillamine is an inhibitor of the latter (56).

In summary, a significant step forward has been achieved by demonstrating that $\text{H}_2\text{O}_2$ is a key factor in the increase in thermal stability of collagen by high glucose in vitro and in vivo. This finding reconciles the previous observations that apparently unrelated treatment modalities may in fact act on $\text{H}_2\text{O}_2$ production or lead to scavenging of oxygen free radicals derived from it. From a therapeutic viewpoint, it is now important to clarify precisely the source of $\text{H}_2\text{O}_2$ production in diabetes and its mechanistic role in protein oxidation and cross-linking.

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