Glycation of type I collagen selectively targets the same helical domain lysine sites as lysyl oxidase–mediated cross-linking

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Nonenzymatic glycation of collagen has long been associated with the progressive secondary complications of diabetes. How exactly such random glycations result in impaired tissues is still poorly understood. Because of the slow turnover rate of most fibrillar collagens, they are more susceptible to accumulate time-dependent glycations and subsequent advanced glycation end-products. The latter are believed to include cross-links that stiffen host tissues. However, diabetic animal models have also displayed weakened tendons with reduced stiffness. Strikingly, not a single experimentally identified specific molecular site of glycation in a collagen has been reported. Here, using targeted MS, we have identified partial fructosyl-hydroxylysine glycation at each of the helical domain cross-linking sites of type I collagen that are elevated in tissues from a diabetic mouse. Glycation of type I collagen selectively targets the same helical domain lysine sites as lysyl oxidase–mediated cross-linking in diabetic tendons. We propose that such N-linked glycations can hinder the normal cross-linking process, thus altering the content and/or placement of mature cross-links with the potential to modify tissue material properties.

Among the known and suspected cross-links in fibrillar collagens, perhaps the least understood but most speculative pathologically are advanced glycation end-products (AGEs). Collagens are well recognized targets for such addition products from glucose and other carbonyl-containing reactants, because they are typically very long-lived proteins with half-lives ranging from 1 to 2 years in bone and 10 years in skin (1) to a 100 years or more in cartilage (2) and tendon (3, 4). It is evident that nonenzymatic AGE cross-links increase over time and are associated with tissue stiffening (5, 6). Remarkably, after decades of research and thousands of publications, no specific sites of AGE cross-linking have yet been experimentally identified in a collagen. There is evidence, however, that fibrillar collagen glycation is not random but likely to occur at favored sites (7).

AGEs derive spontaneously with time by further reactions of initial lysine side-chain glycation adducts. These potentially could include new intra- and intermolecular cross-links (8). Unlike glycosylation (an enzymatically regulated intracellular process during collagen synthesis), which covalently attaches sugar molecules to the hydroxyl moiety of certain hydroxylysines (Hyl), glycation is a nonenzymatic and opportunistic chemical addition of the carbonyl group of free reducing sugars (primarily glucose) to the ε-amine of a Lys side chain. Glycation is also the initial step of the classical Maillard reaction pathway, which begins with a relatively unstable Schiff base adduct of the Lys residue that Amadori rearranges to the more stable ketoimine, fructosyl-Lys. With time these can dehydrate, condense, fragment, and cross-link, thereby forming a complex array of AGES. The most prominent end-product seems to be glucosepane, a fructosyl-Lys to arginine addition product and potential cross-link, formed by dehydration and carbonyl migration along the sugar’s carbon chain. The fructosyl-Lys and an Arg side chain need to be ≤7 Å apart for such a cross-link to form (9). Several potential sites of glucosepane formation in collagen type I fibrils have been predicted theoretically by molecular modeling, but direct proof by tissue analysis is lacking (10, 11).

Hyperglycemia accelerates the age-related decrease in connective tissue quality. One of the biochemical hallmarks of diabetes is tissue stiffening thought to be caused by AGE cross-linking of collagen (12). An accompanying decrease in collagen solubility is consistent with this concept (13). However, reports on the altered material properties of diabetic tendons are inconsistent. For example, conflicting animal model studies have reported both increases (14, 15) and decreases (16–18) in tendon stiffness. Despite decades of research, the pathogenesis of the secondary musculoskeletal effects in diabetes is still poorly understood.

In contrast to stochastic AGE cross-link formation, lysyl oxidase–mediated collagen cross-linking is a biologically con-
controlled and site-specific process that appears to plateau at a relatively young tissue age (19, 20). For example, in type I collagen of mouse tail tendon, the predominant lysyl-oxidase mediated cross-links are acid-labile aldimine bonds. These form in growing fibrils by the addition of telopeptide Lys aldehydes to Hyl at residues 87 or 930/933 in α-chains of adjacent 4D-staggered molecules. In the current study, we examined type I collagen from TallyHo mice, a naturally occurring model of obesity and type 2 diabetes, for glycation sites by peptide MS. The results showed that the same sites targeted by telopeptide Lys residues appeared to be less glycated than their counterparts, with about 14% fructosyl-Hyl identified at α1(I)Lys87 (n = 2) and about 10% α1(I)Lys930 (n = 2) in TallyHo tendon (Fig. 6). In α2(I), glycation levels were 26% (n = 3) in α2(I)Lys90 and 20% (n = 2) in α2(I)Lys933 (Fig. 6). The MS/MS fingerprint of N-linked glycation held true for the current collagenase-digested collagen peptides from TallyHo mouse tendon, in particular at the higher ion charge states (Fig. 2C). Using this mass spectrometric approach, fructosyl-Hyl glycations were identified at α1(I)Lys87, α1(I)Lys930, α2(I)Lys90, and α2(I)Lys933 (Fig. 5). The α1(I) chain helical domain cross-linking Lys residues appeared to be less glycated than their α2(I) chain counterparts, with about 14% fructosyl-Hyl identified at α1(I)Lys87 (n = 2) and about 10% α1(I)Lys930 (n = 2) in TallyHo tendon (Fig. 6). In α2(I), glycation levels were 26% (n = 3) in α2(I)Lys90 and 20% (n = 2) in α2(I)Lys933 (Fig. 6). The MS/MS fingerprint established that residue α2(I)Lys90 was the site of the fructosyl-Hyl, not the candidate cross-linking residue α2(I)Lys87 (Fig. 4C). No alternatively glycated α2(I)Lys97 or doubly glycated α2(I)Lys97+Lys97+ peptides were detected.

Screening the telopeptide cross-linking Lys residues from type I collagen showed no evidence of glycation from diabetic or control mouse tendons, only the peptides with unmodified Lys residues. In addition to nonglycated peptides from the three Lys-containing telopeptide domains, six other peptides containing helical Lys sites were also recovered and found to be nonglycated from diabetic mouse tendon. These unmodified sites included Lys at the Xaa position and Lys/Hyl at the Yaa

Table 1

| Mouse tail tendon | Mass (n = 5) | HbA1c | HP |
|------------------|------------|-------|-----|
| Control          | 33.4 ± 1.5 | 5.0 ± 0.2 | 0.041 ± 0.009 |
| Diabetic          | 36.8 ± 3.2 | 10.2 ± 0.73 | 0.036 ± 0.001 |

Results

Diabetic and control mice

Evidence of increased glycation in TallyHo mouse proteins was associated with elevated HbA1c levels in all 5 diabetic mice (Table 1). All diabetic mice were tested for HbA1c levels at 7% and blood glucose levels >250 mg/dl. Inclusion criteria for control mice (C57BL6) included <6% HbA1c and <200 mg/dl blood glucose. Diabetic mice (36.8 ± 3.2 g; n = 5) were heavier and had notably fattier tissue upon dissection than control mice (33.4 ± 1.5 g; n = 5).

Reduced collagen extractability

Differences in type I collagen extractability in 3% acetic acid from lyophilized tissue samples supported an effect on intermolecular cross-linking in diabetic tendon collagens. Densitometry of stained collagen chains (α, β, and γ) from SDS–PAGE with sample loads normalized to the dry weight of tendon revealed that total collagen was less extractable from TallyHo mouse tail tendons (30%; n = 4) compared with control (Fig. 1, left panel). This decrease in extractability was observed equally across all collagen chains (α, β, and γ). Interestingly, the decrease in extractability was less prominent after pepsin digestion (19%; n = 4) (Fig. 1, right panel). Also notable from the SDS–PAGE band profiles is the lack of any obvious new bands that might arise from new inter-helical stable cross-links introduced as AGEs.

Identification of glycations at collagen cross-linking lysine residues

Type I collagen in mouse tail tendon is well suited for screening for glycation sites because it lacks any enzymatic glycosylation (21, 22). The absence of enzymatic glycosylation facilitates detection of nonenzymatic glycosylation using MS, because both can present as a +162-Da hexose shift. Fortunately, N-glycated Lys residues can be distinguished from O-linked glycosylation in collagen peptides by a unique MS/MS fragmentation pattern, featuring consecutive neutral losses of up to four waters (~18 Da) and one formaldehyde molecule (~30 Da) (23–25). Collision-induced dissociation MS appears to enhance this fingerprint (24). This MS/MS fingerprint of N-linked glycation held true for the current collagenase-digested collagen peptides from TallyHo mouse tendon, in particular at the higher ion charge states (Fig. 2C). Using this mass spectrometric approach, fructosyl-Hyl glycations were identified at α1(I)Lys87 (Fig. 3), α1(I)Lys930 (Fig. 2), α2(I)Lys90 (Fig. 4), and α2(I)Lys933 (Fig. 5). The α1(I) chain helical domain cross-linking Lys residues appeared to be less glycated than their α2(I) chain counterparts, with ~14% fructosyl-Hyl identified at α1(I)Lys87 (n = 2) and ~10% α1(I)Lys930 (n = 2) in TallyHo tendon (Fig. 6). In α2(I), glycation levels were 26% (n = 3) in α2(I)Lys90 and 20% (n = 2) in α2(I)Lys933 (Fig. 6). The MS/MS profile established that residue α2(I)Lys90 was the site of the fructosyl-Hyl, not the candidate cross-linking residue α2(I)Lys87 (Fig. 4C). No alternatively glycated α2(I)Lys97 or doubly glycated α2(I)Lys97+Lys97+ peptides were detected.

Screening the telopeptide cross-linking Lys residues from type I collagen showed no evidence of glycation from diabetic or control mouse tendons, only the peptides with unmodified Lys residues. In addition to nonglycated peptides from the three Lys-containing telopeptide domains, six other peptides containing helical Lys sites were also recovered and found to be nonglycated from diabetic mouse tendon. These unmodified sites included Lys at the Xaa position and Lys/Hyl at the Yaa

Figure 1. SDS–PAGE reveals a decrease in collagen extractability in diabetic mouse tail tendon. Left panel, acid labile aldimine cross-links are broken with mild acetic acid treatment, allowing native type I collagen monomers to be extracted from the tissue. TallyHo mouse tendon was less acid-extractable than control mouse tendon collagen (~30%; n = 4). Right panel, pepsin digestion showed a similar but slightly reduced effect (~19%; n = 4), between control and diabetic tissues. These findings support the presence of AGE cross-links. Sample loads were normalized to the dry weight of tendon prior to loading. Densitometry was performed to quantify stained collagen chains (α, β, and γ) on SDS–PAGE. The disparity between acid and pepsin extraction also supports a possible alteration to acid labile cross-links in TallyHo mouse.

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position of the Gly-Xaa-Yaa repeat domains of the α1(I) and α2(I) chains.

Although Lys/Hyl glycation might be theoretically random in nature, the results clearly show restriction to the helical domain basic motifs that characterize the sites of lysyl oxidase–generated aldehyde interaction. Their percentage occupancy (%) and the sites of glycated Lys residues in type I collagen from diabetic and control mouse tendon are summarized in Fig. 6.

Glycated lysine residues are excluded from normal collagen cross-linking

The fructosyl-Hyl glycations described here and lysyl-oxidase–controlled collagen cross-linking are mutually exclusive processes. Telopeptide Lys aldehydes and reducing sugar carbonyls interact with the ε-amine of helical domain Lys or Hyl residues. Not surprisingly, only nonglycated aldime cross-links were identified in borohydride-reduced diabetic tendon. We were also unable to identify a glycated form of the α2(I)Lys 87–to-C-telopeptide aldime dimeric cross-linked peptide, which might hypothetically have been 30% glycated at α2(I)Lys 90 based on the precursor linear peptide results (Fig. 7). The absence of such a glycated cross-linked peptide suggests that the described glycations are able to both chemically and sterically hinder physiological collagen cross-linking involving telopeptide Lys aldehydes (Fig. 8). In the case of α2(I)Lys 90, glycation cannot occur after a telopeptide has cross-linked to the neighboring α2(I)Lys 87, presumably because of steric hindrance.

Pyridinoline cross-link analysis

As an independent downstream measure of the effect of non-enzymatic glycations on cross-linking Lys residues, pyridinoline cross-links were quantified after acid hydrolysis of mouse tail tendon. Pyridinoline cross-links are only minor cross-links in mouse tail tendon collagens; however, their fluorescence does provide a convenient quantitative index of cross-link content (26). The results revealed a small decrease in hydroxylysyl pyridinoline (HP) cross-links in diabetic tissue (0.036 mol/mol of collagen; \( n = 5 \)) compared with control (0.041 mol/mol of collagen; \( n = 5 \)). This 13% lower HP content in diabetic versus control tendon inversely correlates closely with the increased levels of glycation (~10–20%) at the cross-linking residues (Table 1).

Discussion

Diabetes has become a global pandemic. By recent estimates, over 8% of adults worldwide are affected (27). There is abundant clinical evidence of the adverse effects of hyperglycemia on connective tissues including restricted joint mobility, increased risk of injury, and impaired healing (28–30). An accumulation of AGE cross-links is thought to be an underlying factor in affected tissues (14). In fact, tissue stiffness, mechanical fragi-
Our findings imply that glycation have the potential not only to go on to form AGE-cross-links in proteins but also to chemically hinder normal lysi oxidase-mediated collagen cross-linking. Specifically, glycation of helical domain cross-linking Hyl residues (Lys⁸⁷ and Lys⁹⁰/⁹³) will prevent the modified amino-group from cross-linking to a telopeptide-generated aldehyde. In other words, both collagen telopeptide aldehydes (normal) and glucose carboxyls (abnormal) target the same helical domain cross-linking lysine ε-amine side chains and are therefore mutually exclusive (Figs. 8 and 9). Steric hindrance is also indicated, because α2(I)Lys⁹⁰ glycation apparently physically blocked the neighboring α2(I)Lys⁸⁷ from becoming cross-linked (assuming that in the mouse, α2(I)Lys⁹⁰ is not an alternative binding partner for a telopeptide aldehyde). The absence of a glycated aldimine cross-linked peptide from this site in the collagenase digests supports this interpretation. Diabetic rat tail tendons were previously shown to have significantly less of the reducible collagen cross-link, hydroxylysino Δ norleucine, compared with age-matched control tendon (33). This is consistent with the concept that elevated glycation levels in diabetic collagen can inhibit lysi-oxidase derived cross-link formation. The lower pyridinoline content of hyperglycemic mouse tendon (Table 1) is also consistent with decreased lysi oxidase cross-linking.

Glycations have been shown to increase the mean lateral distance between collagen molecules in tendon fibrils (34), which could distress the supramolecular packing of the fibril structure and so indirectly reduce the cross-linking potential. Also, if glycation was most concentrated on collagen molecules at fibril surfaces, as is likely from size-exclusion considerations, this could prevent newly made collagen molecules from cross-linking to existing fibrils during tissue growth and remodeling. Aldimine cross-links and free Lys aldehydes are likely to be in a continual state of equilibrium on the surface of collagen fibrils, even in mature tendons. Indeed, it has been demonstrated in tendon construct cultures that inhibiting lysi oxidase activity after 1 month in culture resulted in mechanical failure of the entire fabric (35).

The current study also adds support to a proposed association between glycation and tissue stiffening. An observed decrease in collagen extractability from diabetic tendon is consistent with the notion that glycation goes on to introduce intermolecular AGE cross-links (13, 33). The decrease in collagen extractability implies an increase in acid-stable intermolecular cross-links. The smaller effect on collagen solubility upon pepsin digestion compared with acid extraction supports the addition of inter-triple-helical cross-links in diabetic mouse tendon (although no evidence was noted for such bonds in the form of new bands in Fig. 1). Accumulated AGE cross-links have been associated with both increased (14, 15) and decreased (16 –18) tendon stiffening. However, there is also other evidence to indicate that AGES do not affect individual collagen fibril stiffness (36) but may, in fact, significantly dimin-

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ish overall tendon viscoelasticity (37). Nevertheless, it is clear that factors that impact collagen synthesis, turnover, or enzymatic cross-linking can also impact tissue mechanical properties. A reduced number of lysyl oxidase–mediated cross-links coupled with an increase in AGE cross-links, dependent on where the latter were placed, could have the net effect of both reducing the strength and increasing the stiffness of tendon.

Non-Glycated Cross-linking Lysines

**Sequence**
- α1(1) GFSGLDGAKGDAGPA (Lys99)
- α1(1) GPPGAVPGAKDGDEAGAQAP (Lys434)
- α1(1) GKDLGLNLGP (Lys974)
- α2(1) GFGSNGVPSPGKEGVPGLPGIDGRP (Lys374)
- α2(1) GPGQVGQGGKGEQGPA (Lys453)
- α2(1) GPSGGTGEQVKGERGLPGFGLP (Lys579)

Non-Glycated Helical Lysines

**Sequence**
- α1(1) GFSGLDGAKGDAGPA (Lys99)
- α1(1) GPPGAVPGAKDGDEAGAQAP (Lys434)
- α1(1) GKDLGLNLGP (Lys974)
- α2(1) GFGSNGVPSPGKEGVPGLPGIDGRP (Lys374)
- α2(1) GPGQVGQGGKGEQGPA (Lys453)
- α2(1) GPSGGTGEQVKGERGLPGFGLP (Lys579)

Figure 5. Fructosyl-hydroxylysine at the α2(1)Lys^{933} collagen cross-linking site in diabetic tendon. LC–MS profiles of collagenase-digested TallyHo and control mouse tendon are shown. A, C57BL6 mouse tendon shows minimal glycination of the α2(1)Lys^{933}-containing peptide (611.7^2+). B, this cross-linking residue from diabetic mouse tendon is significantly glycated (~20% fructosyl-HyL; 692.7^2+). C, MS/MS confirms the amino acid sequence of the Hyl-containing peptide (611.7^2+). D, MS/MS profile with neutral losses of water (H₂O; 18 Da) confirms the amino acid sequence of the glycated peptide (692.7^2+). The blue hexagon indicates fructosyl-moiety; P* and K* indicate 4Hyp and Hyl, respectively.

Figure 6. Glycation of helical domain collagen cross-linking hydroxylysine residues is elevated in diabetic mouse. The percentages of fructosyl-Hyl at the helical domain cross-linking sites of type I collagen from tendon (n = 2) are shown. The percentages were determined based on the ratio of the m/z peaks of each post-translational variant as previously described (47). It should be noted that in mouse, α2(1)Lys^{933} is glycated rather than α2(1)Lys^{900}. In rat and human, there is no Lys at position 90. Glycation of helical domain cross-linking residues appears to be selective, because all other peptides we recovered by LC–MS that contained Lys and/or Arg residues from the triple helical and telopeptide regions of both α-chains proved to be nonglycated. J indicates pyroglutamic acid.
entially with glucose (38). Nearby negative carboxyls on Glu or Asp are predicted to lower the pK_a of Lys residues (38) and mechanistically help catalyze the subsequent Amadori rearrangement by stabilizing the Schiff base intermediate. Nearby basic residues (Lys, Arg, and His) also promote Lys residue glycation (39). As candidate glycation sites, the helical domain cross-linking Lys residues are ideally located adjacent to Lys, Arg, and His residues. This consensus sequence is evident at every helical domain cross-linking site (mouse sequences shown): α1(I)Lys^{87} (GMKKGHR); α1(I)Lys^{330} (GDRGIKGR); α2(I)Lys^{87} (GFKGVKGR HS); and α2(I)Lys^{893} (GHRGLPGKL). Clearly, the same local factors that have evolved to favor telopeptide aldehyde additions also favor glucose carbonyl additions.

Simulations of atomistic models have recently been used to predict theoretical sites of AGE cross-linking in collagen (10, 11, 40). In these studies, both α1(I)Lys^{87} (10) and α2(I)Lys^{87} (11) came up as prominent predicted sites of glycation. Our results show that α2(I)Lys^{90} was favorably glycated over cross-linking residue Lys^{87}; however, this Lys (GFKGVKGH) appears to be unique to the mouse sequence and in other higher mammals, including rat and humans, is replaced by Arg (GFKIRGHN). We have evidence that supports α2(I)Lys^{87} being the residue glycated in higher mammals other than mouse (data not shown).

Site-specific glycations have been proposed for other proteins, including collagens (7, 38, 39, 41). From rat tail tendon, CNBr-digested collagen peptides α2(I)CB3.5 and α1(I)CB3 were studied for glycations (7). Four sites with trace levels of glycation (less than 1% occupancy each) were identified after radiolabeling by reduction with tritiated sodium borohydride (α1(I)Lys^{434}, α2(I)Lys^{453}, α2(I)Lys^{579}, and α2(I)Lys^{929}). These findings support our conclusion that Lys sites other than those identified in the present study are only negligibly glycated. Indeed, the same residues noted in the above study (α1(I)Lys^{434}, α2(I)Lys^{453}, and α2(I)Lys^{579}) were identified in the TallyHo tendon collagenase digests and found to be non-glycated (<1%) by the current mass spectrometric approach (Fig. 6).

Protein glycation is a rapid and, initially, potentially reversible reaction (42), with the degree of glycation being proportional to the level of glucose in the blood. The product from glucose (fructosyl-Lys) can break down to carboxymethyl-Lys, which has been predicted to cause tissue damage through its ability to chelate transitional metals (43). The latter AGE can also produce reactive oxygenated species (43). Unlike the initial glycation product, the predicted AGE cross-links are not reversible (8). In future studies, it will be important to identify specific sites of the major AGES, such as glucosepane predicted to bridge Lys and Arg side chains, and determine whether any are intermolecular and therefore potentially detrimental to tissue properties. It will also be important to understand at the level of the fibril and suprafibrillar architecture whether glycation is unevenly distributed, for example concentrated on the surface more than the interior of fibrils.

### Materials and methods
#### Diabetic mice

All animal work was performed with institutional approval by the University of Colorado Anschutz Medical Center Institutional Animal Care and Use Committee. Eight-week-old TallyHo mice (n = 5) (TALLYHO/JngJ; stock no. 005314) and control mice (n = 5) (C57BL6/J; stock no. 000664) were purchased from The Jackson Laboratory (Bar Harbor, ME). TallyHo mice are a naturally occurring polygenic model of obesity and type 2 diabetes derived from the selective breeding of hyperglycemia in mice over multiple generations. Only male mice were used in this study (the disease is not penetrant in females). The animals were kept under standard conditions until euthanasia at 26 weeks. The animals were maintained on a standard chow diet (catalogue no. 2920X; Envigo, Madison, WI), sterilized by irradiation, and given free access to food and water. Body mass was measured weekly. Blood samples were drawn to measure blood glucose and HbA1c levels at 17 weeks of age and at euthanasia. Inclusion criteria for the study included HbA1c levels (>7%) and blood glucose levels (>250 mg/dl). The study was not blinded (TallyHo mice are white, and the C57BL/6J mice are black).

#### Collagen extraction

Tail tendon collagens were characterized in TallyHo (test) and C57BL6 (control) mice. Intact type I collagen was solubilized from the tissues by acid extraction in 3% acetic acid for 24 h at 4 °C. Total collagen was extracted by limited pepsin digestion. Tissues were also digested with bacterial collagenase with and without borohydride reduction, and total collagenase digests were resolved into peptide fractions by C8 reverse-phase HPLC (44). Acid-extracted and pepsin-extracted colla-
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Figure 9. Model of altered collagen cross-link formation in a glycated tendon. A, in the fibril, collagen molecules are spatially staggered in an arrangement that promotes ordered intermolecular cross-linking. B, in normal mouse tendon, type I collagen initially forms intermolecular aldimine cross-links between helical domain Hyl and telopeptide Lys aldehydes. In diabetic collagen, partially glycated helical domain cross-linking Hyl are sterically and/or chemically hindered from participating in normal collagen cross-linking chemistry. The net effect could be a tendon with compromised material properties resulting from fewer physiologically stable cross-links yet subsequent tissue stiffening from potential inappropriately placed AGE cross-links in the fibrillar matrix architecture.

gen α-chains were resolved by SDS–PAGE and stained with Coomassie Blue R-250. Collagen extractability was determined based on band intensities using National Institutes of Health ImageJ software as previously described (45).

Collagen cross-linking analysis

The collagen pyridinoline cross-link content was determined by fluorescence monitoring with reverse-phase HPLC. Pyridinoline cross-links were analyzed in mouse tail tendon by HPLC after acid hydrolysis in 6 M HCl for 24 h at 108 °C. Dried samples were dissolved in 1% (v/v) n-heptfluorobutyric acid for quantitation of HP by reverse-phase HPLC and fluorescence monitoring as previously described (46).

Mass spectrometry

Glycations (fructosyl-Lys and fructosy-Hyl) and glycosylations (glucosylgalactosyl-Hyl and galactosyl-Hyl) were quantified at specific sites in collagen α-chains as previously described (47). Collagen α-chains were cut from SDS–PAGE gels and subjected to in-gel trypsin digestion. Tendons were also digested with bacterial collagenase, with and without borohydride reduction, and resolved by C8 reverse-phase HPLC prior to analysis by MS (44, 48). Electrospray MS was carried out on the trypsin- and collagenase-digested peptides using an LTQ XL linear quadrupole ion-trap mass spectrometer equipped with in-line Accela 1250 LC and automated sample injection (ThermoFisher Scientific). Proteome Discoverer software (ThermoFisher Scientific) was used for peptide identification. Tryptic peptides were also identified manually by calculating the possible MS/MS ions and matching them to the actual MS/MS spectrum using Thermo Xcalibur software. Differences in post-translational modifications were determined manually by averaging the full scan MS over several LC–MS minutes to include all the post-translational variations of a given peptide. Protein sequences used for MS analysis were obtained from the Ensembl genome database.

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References

1. Avery, N. C., and Bailey, A. J. (2006) The effects of the Maillard reaction on the physical properties and cell interactions of collagen. Pathol. Biol. 54, 387–395 CrossRef Medline
2. Verzijl, N., DeGroot, J., Thorpe, S. R., Bank, R. A., Shaw, J. N., Lyons, T. J., Bijlsma, J. W., Lafeber, F. P., Baynes, J. W., and Tekoppele, J. M. (2000) Effect of collagen turnover on the accumulation of advanced glycation end products. J. Biol. Chem. 275, 39027–39031 CrossRef Medline
3. Thorpe, C. T., Streeter, I., Pinchbeck, G. L., Godshield, A. E., Clegg, P. D., and Birch, H. L. (2010) Aspartic acid racemization and collagen degradation markers reveal an accumulation of damage in tendon collagen that is enhanced with aging. J. Biol. Chem. 285, 15674–15681 CrossRef Medline
4. Heinemeier, K. M., Schjerling, P., Heinemeier, J., Magnusson, S. P., and Kjaer, M. (2013) Lack of tissue renewal in human adult Achilles tendon is revealed by nuclear bomb 14C. FASEB J. 27, 2074–2079 CrossRef Medline
5. Bank, R. A., Bayliss, M. T., Lafefer, F. P., Maroudas, A., and Tekoppele, J. M. (1998) Ageing and zonal variation in post-translational modification of collagen in normal human articular cartilage. The age-related increase in non-enzymatic glycation affects biomechanical properties of cartilage. Biochem. J. 330, 345–351 CrossRef Medline
6. Snedeker, J. G., and Gautieri, A. (2014) The role of collagen crosslinks in ageing and diabetes: the good, the bad, and the ugly. Muscles Ligaments Tendons J. 4, 303–308 Medline
7. Reiser, K. M., Amigable, M. A., and Last, J. A. (1992) Nonenzymatic glycation of type I collagen. The effects of aging on preferential glycation sites. J. Biol. Chem. 267, 24207–24216 Medline
8. Monnier, V. M., Mustata, G. T., Biemel, K. L., Rehl, O., Lederer, M. O., Zhengy, D., and Sell, D. R. (2005) Cross-linking of the extracellular matrix by the maillard reaction in aging and diabetes: an update on “a puzzle nearing resolution”. Ann. N.Y. Acad. Sci. 1043, 533–544 CrossRef Medline
9. Dai, Z., Wang, B., Sun, G., Fan, X., Anderson, V. E., and Monnier, V. M. (2008) Identification of glucose-derived cross-linking sites in ribonuclease A. J. Proteome Res. 7, 2756–2768 CrossRef Medline
10. Gautieri, A., Redaelli, A., Buehler, M. J., and Vesentini, S. (2014) Age- and diabetes-related nonenzymatic crosslinks in collagen fibrils: candidate amino acids involved in advanced glycation end-products. Matrix Biol. 34, 89–95 CrossRef Medline
11. Collier, T. A., Nash, A., Birch, H. L., and de Leeuw, N. H. (2015) Preferential sites for intramolecular glucoseamine cross-link formation in type I collagen: a thermodynamic study. Matrix Biol. 48, 78–88 CrossRef Medline
12. Brownlee, M., Vlassara, H., Kooney, A., Ulrich, P., and Cerami, A. (1986) Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking. Science 232, 1629–1632 CrossRef Medline
Nonenzymatic collagen glycation sites

13. Brennan, M. (1989) Changes in solubility, non-enzymatic glycation, and fluorescence of collagen in tail tendons from diabetic rats. J. Biol. Chem. 264, 20947–20952 CrossRef Medline

14. Andreasen, T. T., Seyer-Hansen, K., and Bailey, A. J. (1981) Thermal stability, mechanical properties and reducible cross-links of rat tail tendon in experimental diabetes. Biochim. Biophys. Acta 677, 313–317 CrossRef Medline

15. McLennan, S., Yue, D. K., Marsh, M., Swanson, B., Delbridge, L., Reeve, T., and Turtle, J. R. (1986) The prevention and reversibility of tissue non-enzymatic glycosylation in diabetes. Diabet. Med. 3, 141–146 CrossRef Medline

16. de Oliveira, R. R., de Lira, K. D., Silveira, P. V., Coutinho, M. P., Medeiros, M. N., Teixeira M. F., and de Moraes, S. R. (2011) Mechanical properties of achilles tendon in rats induced to experimental diabetes. Ann. Biomed. Eng. 39, 1528–1534 CrossRef Medline

17. Fox, A. J., Bedi, A., Deng, X.-H., Ying, L., Harris, P. E., Warren, R. F., and Rodeo, S. A. (2011) Diabetes mellitus alters the mechanical properties of the native tendon in an experimental rat model. J. Orthop. Res. 29, 880–885 CrossRef Medline

18. Connizzo, B. K., Bhatt, P. R., Liechty, K. W., and Soslovsy, L. J. (2014) Diabetes alters mechanical properties and collagen fiber re-alignment in multiple mouse tendons. Ann. Biomed. Eng. 42, 1880–1888 CrossRef Medline

19. Haut, R. C., Lancaster, R. L., and DeCamp, C. E. (1992) Mechanical properties of the canine patellar tendon: some correlations with age and the content of collagen. J. Biomech. 25, 163–173 CrossRef Medline

20. Eyre, D. R., Dickson, I. R., and Van Ness, K. (1988) Collagen cross-linking in human bone and articular cartilage. Age-related changes in the content of mature hydroxyprolylum residues. Biochem. J. 252, 495–500 CrossRef Medline

21. Hudson, D. M., Garibov, M., Dixon, D. R., Popowics, T., and Eyre, D. R. (2017) Distinct post-translational features of type I collagen are conserved in mouse and human periodontal ligament. J. Periodontal. Res. 52, 1042–1049 CrossRef Medline

22. Hudson, D. M., Weis, M., Rai, J., Joeng, K. S., Dimori, M., Lee, B. H., Morello, R., and Eyre, D. R. (2017) P3h3-null and Sc65-null mice phenotype the collagen lysine under-hydroxylation and cross-linking abnormality of Ehlers-Danlos syndrome type vIA. J. Biol. Chem. 292, 3877–3887 CrossRef Medline

23. Frolov, A., Hoffmann, P., and Hoffmann, R. (2006) Fragmentation behavior of glycated peptides derived from d-glucose, d-fructose and d-ribose in tandem mass spectrometry. J. Mass Spectrom. 41, 1459–1469 CrossRef Medline

24. Frolov, A., and Hoffmann, R. (2010) Identification and relative quantification of specific glycation sites in human serum albumin. Anal. Bioanal. Chem. 397, 2349–2356 CrossRef Medline

25. Horvat, S., and Jakas, A. (2004) Peptide and amino acid glycation: new insights into the Maillard reaction. J. Pept. Sci. 10, 119–137 CrossRef Medline

26. Eyre, D. R., Paz, M. A., and Gallop, P. M. (1984) Cross-linking in collagen and elastin. Annu. Rev. Biochem. 53, 717–748 CrossRef Medline

27. Global Status Report on Noncommunicable Diseases 2014 (2015) World Health Organization, Geneva, Switzerland

28. Stolarczyk, A., Sarzyńska, S., Gondek, A., and Cudnoch-Jdrzejewska, A. (2018) Influence of diabetes on tissue healing in orthopedic injuries. Clin. Exp. Pharmacol. Physiol. 45, 619–627 CrossRef Medline

29. Hamann, C., Kirschner, S., Günther, K.-P., and Hoßbauer, L. C. (2012) Bone, sweet bone–osteoporotic fractures in diabetes mellitus. Nat. Rev. Endocrinol. 8, 297–305 CrossRef Medline

30. Semba, R. D., Nicklett, E. J., and Ferrucci, L. (2010) Does accumulation of advanced glycation end products contribute to the aging phenotype? J. Gerontol. A Biol. Sci. Med. Sci. 65, 963–975 CrossRef Medline

31. Galeski, A., Kastelic, J., Baer, E., and Kohn, R. R. (1977) Mechanical and structural changes in rat tail tendon induced by alloxan diabetes and aging. J. Biomech. 10, 775–782 CrossRef Medline

32. Sobeleva, A., Schmidt, R., Vikhina, M., Grishina, T., and Frolov, A. (2017) Maillard proteomics: opening new pages. Int. J. Mol. Sci. 18, E2677 CrossRef Medline

33. Brennan, M. (1989) Changes in the cross-linking of collagen from rat tail tendons due to diabetes. J. Biol. Chem. 264, 20953–20960 CrossRef Medline

34. James, V. J., Delbridge, L., McLennan, S. V., and Yue, D. K. (1991) Use of X-ray diffraction in study of human diabetic and aging collagen. Diabetes 40, 391–394 CrossRef Medline

35. Herchenhan, A., Uhlenbrock, F., Eliaison, P., Weis, M., Eyre, D., Kraler, K. E., Magnusson, S. P., and Kjaer, M. (2015) Lysyl oxidase activity is required for ordered collagen fibrillogenesis by tendon cells. J. Biol. Chem. 290, 16460–16450 CrossRef Medline

36. Fessel, G., Li, Y., Diederich, V., Guizar-Sicario, M., Schneider, P., Sell, D. R., Monnier, V. M., and Snedeker, J. G. (2014) Advanced glycation end-products reduce collagen molecular sliding to affect collagen fibril damage mechanisms but not stiffness. PLoS One 9, e110948 CrossRef Medline

37. Li, Y., Fessel, G., Georgiadis, M., and Snedeker, J. G. (2013) Advanced glycation end-products diminish tendon collagen fiber sliding. Matrix Biol. 32, 169–177 CrossRef Medline

38. Shapiro, R., McManus, M. J., Zalut, C., and Bunn, H. F. (1980) Sites of nonenzymatic glycosylation of human hemoglobin A. J. Biol. Chem. 255, 3120–3127 Medline

39. Watkins, N. G., Thorpe, S. R., and Baynes, J. W. (1985) Glycation of amino groups in protein. Studies on the specificity of modification of RNase by glucose. J. Biol. Chem. 260, 10629–10636 Medline

40. Collier, T. A., Nash, A., Birch, H. L., and de Leeuw, N. H. (2016) Intramolecular lysine-arginine derived advanced glycation end-product cross-linking in type I collagen: a molecular dynamics simulation study. Biophys. Chem. 218, 42–46 CrossRef Medline

41. Iberg, N., and Flückiger, R. (1986) Nonenzymatic glycosylation of albumin in vivo: Identification of multiple glycosylated sites. J. Biol. Chem. 261, 13542–13545 Medline

42. Sell, D. R., and Monnier, V. M. (2012) Molecular basis of arterial stiffening: role of glycation: a mini-review. Gerontology 58, 227–237 CrossRef Medline

43. Saxena, A. K., Saxena, P., Wu, X., Obrenovich, M., Weiss, M. F., and Monnier, V. M. (1999) Protein aging by carboxymethylation of lysines generates sites for divalent metal and redox active copper binding: relevance to diseases of glycoxidative stress. Biochem. Biophys. Res. Commun. 260, 332–338 CrossRef Medline

44. Heard, M. E., Besio, R., Weis, M., Rai, J., Hudson, D. M., Dimori, M., Zimmerman, S. M., Kamykowskii, J. A., Hogue, W. R., Swan, F. L., Burdine, M. S., Mackintosh, S. G., Tackett, A. J., Suva, L. J., Eyre, D. R., et al. (2016) Sc65-null mice provide evidence for a novel endoplasmic reticulum complex regulating collagen hylx hydration. PLoS Genet. 12, e1006002 CrossRef Medline

45. Kalamajski, S., Liu, C., Tillgren, V., Rubin, K., Oldberg, Å., Rai, J., Weis, M., and Eyre, D. R. (2014) Increased C-telopeptide cross-linking of tendon type I collagen in fibromodulin-deficient mice. J. Biol. Chem. 289, 18877–18879 CrossRef Medline

46. Eyre, D. (1987) Collagen cross-linking amino acids. Methods Enzymol. 144, 115–139 CrossRef Medline

47. Weis, M. A., Hudson, D. M., Kim, L., Scott, M., Wu, J. I., and Eyre, D. R. (2010) Location of 3-hydroxyproline residues in collagen types I, II, III, and V/XI implies a role in fibril supramolecular assembly. J. Biol. Chem. 285, 2580–2590 CrossRef Medline

48. Hanson, D. A., and Eyre, D. R. (1996) Molecular site specificity of pyridinoline and pyrrolo-cross-links in type I collagen of human bone. J. Biol. Chem. 271, 26508–26516 CrossRef Medline