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Analysis of Advanced Glycation Endproducts in Rat Tail Collagen and Correlation to Tendon Stiffening

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ABSTRACT: Methylglyoxal is a major 1,2-dicarbonyl compound in vivo and leads to nonenzymatic protein modifications, known as advanced glycation endproducts. Especially long-lived proteins like collagen are prone to changes of the mechanical or biological function, respectively, by accumulation of Maillard-derived modifications. Specifically, the resulting nonenzymatic cross-link structures in parallel to the natural maturation process of collagen fibrils lead to complications with age or during disease. A novel lysine−lysine amide cross-link derived from methylglyoxal, 2,15-diamino-8-methyl-9-oxo-7,10-diaza-1,16-hexadecanedioic acid, named MOLA, was synthesized and identified in vitro and in vivo. Tail tendons of young, adult, and old rats (3, 12, and 22 months) were enzymatically digested prior to analysis of acid-labile glycation products via liquid chromatography−tandem mass spectrometry (LC−MS/MS). As a result, nine monovalent amino acid modifications, mostly originating from methylglyoxal (36 μmol/mol leucine-equivalents in total), and four glycation cross-links (0.72 μmol/mol glucosepane, 0.24 μmol/mol DODIC (3-deoxyglucosone-derived imidazoline cross-link), 0.04 μmol/mol MODIC (methylglyoxal-derived imidazoline cross-link), 0.34 μmol/mol MOLA) were quantitated in senescent tendon collagen. The results correlated with increased tail tendon breaking time from 10 to 190 min and indicate that methylglyoxal is a major player in the aging process of connective tissue.

KEYWORDS: *methylglyoxal, Maillard reaction, advanced glycation endproducts, collagen, glycation cross-links, connective tissue stiffening*

INTRODUCTION

The Maillard reaction or glycation is one crucial aspect of protein modification during aging in vivo. In complex reaction cascades reducing sugars, especially 1,2-dicarbonyl compounds, interact with functional groups of lysine and arginine side chains to form stabile amino acid modifications, named advanced glycation endproducts (AGEs).[1] Long-lived proteins of the extracellular matrix, with a turnover of several years, are highly susceptible to structural changes due to accumulation of AGEs over time resulting in tissue stiffening and dysfunction.[2] Collagen is the most abundant polypeptide in connective tissues (e.g., tendon, skin, bone, cartilage, or vessels). A total of 26 different types of collagen with a broad functional diversity exist.[3] The fibril-forming type I collagen, mainly found in skin and tendons, is an established model for both in vitro and in vivo studies due to its structural homology among different organisms and, thus, has been studied intensively. Glycation cross-link structures are assumed to contribute and to explain collagen damage on a molecular basis.[4,5] However, only a few selected structures, specifically N^6-carboxymethyl lysine (CML), N^6-carboxyethyl lysine (CEL), MG-H1, and pentosidine, were reported in literature as collagen modifications in vivo.[6,7] In more recent work, glucosepane was considered as a major glycation cross-link in extracellular matrix protein.[5,8]

One very potent glycating agent in biological systems is methylglyoxal (MGO). MGO is a short-chained, highly reactive 1,2-dicarbonyl compound originating from numerous sources in vivo including the Maillard triggered breakdown of longer-chained sugars but foremost the enzymatic and nonenzymatic degradation of triose phosphates.[9] Quantitation of MGO in vivo was conducted comprehensively. Reported MGO levels in human plasma range from 100 to 500 nM in healthy nondiabetic subjects,[10] while in rats plasma levels are around 2 μM.[11] In cells and tissues, MGO levels are commonly 2−4 μM.[12] Until now, nine MGO-related amino acid derivatives are reported in the literature. Lysine modifications are CEL and N^6-lactoyl lysine.[13,14] The methylglyoxal hydroimidazolones MG-H3 and MG-H1, their open-chained form N^7-carboxyethyl arginine (CEA) and the two pyrimidine structures tetrahydropyrimidine (THP) and argpyrimidine are derived from arginine.[15] Two cross-link structures are known to originate from MGO, the acid-stable lysine−lysine imidazolium compound MOLD (methylglyoxal lysine dimer),[16] and the acid-labile lysine-arginine amidine MODIC (methylglyoxal-derived imidazoline cross-link).[17] The aim of the present work was therefore to gain further insights into the mechanisms of AGE formation in collagen and to highlight relevant structures with a focus on MGO chemistry. Correlation of degree of chemical modification to change of physical properties was first accessed in an ex vivo model using 3 months old rat tail tendons incubated with methylglyoxal (200 μM) under physiological conditions (pH 7.4, 37 °C) for 7 days. The study was then extended to the natural aging process using native tendons of

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young, adult and old rats (3, 12, and 22 months). A novel MGO-derived amide AGE cross-link structure was identified and quantitated in parallel to other established AGEs.

MATERIALS AND METHODS

Chemicals. All chemicals of the highest quality available were purchased from Sigma-Aldrich (Munich/Steinheim, Germany) and Carl Roth AG (Karlsruhe, Germany) unless otherwise indicated. Methylglyoxal was prepared freshly from its dimethyl acetal as described by Klöpfer et al.[15] N^6-carboxymethyl lysine (CML),[18] N^6-glycoloyl lysine (GALA),[19] N^7-carboxymethyl arginine (CMA),[20] glyoxal hydroimidazolone (G-H3),[21] N^6-carboxyethyl lysine (CEL),[13] N^6-lactoyl lysine,[14] and argpyrimidine[22] were synthesized according to literature. Methylglyoxal hydroimidazolones (MG-H1 and MG-H3) as well as N^7-carboxyethyl arginine (CEA) and tetrahydropyrimidine (THP) were isolated from methylglyoxal/N^2-*t*-Boc-arginine reaction mixtures as described previously by our working group.[15] Preparation of glucosepane,[23] MODIC (methylglyoxal-derived imidazoline cross-link)[17] and DODIC (3-deoxyglucosone-derived imidazoline cross-link)[24] was carried out as described by Lederer's group.

Synthesis of MOLA. The basic synthetic route was adapted with modifications from Glomb and Pfahler.[19] Thin-layer chromatography (TLC) was performed on silica gel 60 F254 (Merck, Germany). Visualization of separated material was achieved with ninhydrin (2% ninhydrin + 5% acetic acid in *n*-butanol, w/v/v). Preparative column chromatography was performed on silica gel 60 (0.06−0.20 mm, Merck, Germany). Solvents were all chromatographic grade. From the individual fractions, solvents were removed under reduced pressure.

*t-Butyl-N^2-(t-butyloxycarbonyl)-N^6-(benzyloxycarbonyl)-L-lysinate (**2**).* **2** was synthesized according to Strazzolini et al.:[25] 2.0 g (5.26 mmol) of N^2-(*t*-butyloxycarbonyl)-N^6-(benzyloxycarbonyl)-L-lysine (**1**) and 330 mg (2.70 mmol) of 4-dimethylaminopyridine were dissolved in 10 mL of warm *t*-butanol. 1222 mg (5.60 mmol) of di-*t*-butyl dicarbonate (Boc_2O) were added. The solution was stirred for 24 h at room temperature (RT). Solvents were evaporated and the resulting residue was subjected to column chromatography (silica gel, *n*-hexane/acetone, 3:1, v/v). Fractions with an R_f value of 0.38 were combined (TLC, same eluent), and solvents were evaporated to yield **2** as a colorless oil (2.2 g, 95%).

*t-Butyl-N^2-(t-butyloxycarbonyl)-L-lysinate (**3**).* 2.2 g (5.01 mmol) of **2** were dissolved in 10 mL of methanol/dichloromethane (1:1) with a small amount of palladium/activated charcoal (10%). The stirred mixture was purged with hydrogen for 2 h. The reaction was monitored by TLC (R_f 0.24, silica gel, methanol/dichloromethane/triethylamine, 70:29:1, v/v/v). After filtration through Celite, solvents were evaporated to yield **3** as a brown oil (1.4 g, 93%).

*t-Butyl-N^2-(t-butyloxycarbonyl)-N^6-(carboxyethyl)-L-lysinate (**4**).* 700 mg (2.31 mmol) of **3** and 203 mg (2.31 mmol) of pyruvic acid were dissolved in 5 mL of absolute methanol. After addition of 435 mg (6.93 mmol) of sodium cyanoborohydride, the solution was stirred at RT for 3 h. Solvents were evaporated and the resulting residue was subjected to column chromatography (silica gel, methanol/ethyl acetate, 30:70, v/v). Fractions with material having an R_f value of 0.24 were combined and solvents were evaporated to yield **4** as a white powder (450 mg, 52%).

HR-MS: m/z 373.2339 $[M - H]^-$ (found), m/z 373.2344 (calculated for $C_{18}H_{33}N_2O_6$ $[M - H]^-$).

^{13}C NMR (100 MHz, CD_3OD): δ [ppm] = 174.4, 173.5, 158.1, 82.6, 80.5, 59.2, 55.5, 47.1, 32.1, 28.7, 28.3, 27.0, 24.1, 16.2.

^1H NMR (500 MHz, CD_3OD): δ [ppm] = 4.00−3.91 (m, 1H), 3.52 (q, J = 7.2 Hz, 1H), 3.05−2.90 (m, 2H), 1.85−1.48 (m, 6H), 1.48 (d, J = 7.2 Hz, 3H), 1.46 (s, 9H), 1.44 (s, 9H).

*t-Butyl-N^2,N^6-bis(t-butyloxycarbonyl)-N^6-(carboxyethyl)-L-lysinate (**5**).* 450 mg (1.20 mmol) of **4** were dissolved in 10 mL of a triethylamine solution (10% in absolute methanol, v/v). 1310 mg (6.00 mmol) of Boc_2O were added and the reaction mixture was refluxed for 30 min while stirring. Stirring was continued for 30 min at RT, solvents were evaporated, and the resulting residue was taken up in ice-cold 0.5 N hydrochloric acid. The emulsion was immediately extracted with ethyl acetate. The organic layer was dried with sodium sulfate, solvents were evaporated, and the residue was subjected to column chromatography (silica gel, *n*-hexane/acetone, 80:20, v/v). Fractions with material having an R_f value of 0.10 were combined and solvents were evaporated to yield **5** as a colorless oil (301 mg, 53%).

HR-MS: m/z 473.2864 $[M - H]^-$ (found), m/z 473.2868 (calculated for $C_{23}H_{41}N_2O_8$ $[M - H]^-$).

^{13}C NMR (100 MHz, CD_3OD) δ [ppm] = 175.6, 173.8, 158.1, 154.3, 83.4, 82.5, 80.4, 57.2, 55.8, 47.6, 32.4, 28.8, 28.7, 28.3, 28.0, 24.0, 17.4.

^1H NMR (500 MHz, CD_3OD) δ [ppm] = 4.03 (q, J = 7.0 Hz, 1H), 3.94 (s, 1H), 3.30−3.04 (m, 2H), 1.82−1.51 (m, 6H), 1.47 (d, J = 2.0 Hz, 3H), 1.47 (s, 9H), 1.45 (s, 18H).

*1,16-Di(t-butyl)-2,15-di[(t-butyloxycarbonyl)amino]-7-(t-butyloxycarbonyl)-8-methyl-9-oxo-7,10-diazahexadecanedioate (**6**).* 100 mg (0.21 mmol) of **5** were dissolved in 2 mL of absolute tetrahydrofuran (THF) and 41 mg (0.30 mmol) of 1-hydroxybenzotriazole were added while stirring. 47 mg (0.30 mmol) of 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide dissolved in 1 mL of THF were added dropwise at 0 °C. After 30 min 64 mg (0.21 mmol) of **3** dissolved in 2 mL of THF were added to the solution. The reaction mixture was stirred for 24 h at room temperature. Solvents were evaporated and the resulting residue was subjected to column chromatography (silica gel, *n*-hexane/acetone, 70:30, v/v). Fractions with an R_f value of 0.43 were combined and solvents were evaporated to yield **6** as a colorless oil (150 mg, 95%).

HR-MS: m/z 759.5121 $[M + H]^+$ (found), m/z 759.5114 (calculated for $C_{38}H_{71}N_4O_{11}$ $[M + H]^+$).

*2,15-Diamino-8-methyl-9-oxo-7,10-diaza-1,16-hexadecanedioic acid (MOLA, **7**).* 100 mg (0.13 mmol) of **6** were dissolved in 2 mL of THF and 2 mL of hydrochloric acid (6.0 M) were added. The reaction was monitored by TLC (silica gel, *n*-butanol/water/acetic acid/pyridine, 4:2:3:3, v/v/v/v). Solvents were removed under reduced pressure. The product was dried over potassium hydroxide under high vacuum to yield **7** as a brown, amorphic material (44 mg, 98%, MOLA × 3 HCl).

HR-MS: m/z 345.2141 $[M-H]^-$ (found), m/z 345.2143 (calculated for $C_{15}H_{29}N_4O_5$ $[M-H]^-$).

^{13}C NMR (100 MHz, D_2O): δ [ppm] = 172.99, 172.89, 170.48, 170.39, 57.21, 53.88, 53.76, 46.76, 40.03, 39.90, 30.43, 30.29, 28.74, 28.72, 26.17, 22.58, 22.49, 16.72.

^1H NMR (500 MHz, D_2O): δ [ppm] = 4.14−4.07 (m, 2H), 3.98 (q, J = 7.0 Hz, 1H), 3.37−3.21 (m, 2H), 3.14−2.94 (m, 2H), 2.09−1.89 (m, 4H), 1.84−1.73 (m, 2H), 1.67−1.38 (m, 6H), 1.53 (d, J = 7.0 Hz, 3H).

Animals. Three and 12 months old male Wistar rats were bred in the Center of Medical Basic Research (ZMG), Medical Faculty, Martin-Luther-University (Halle, Germany). Eighteen months old male Wistar rats were purchased from Janvier Laboratories (Le Genest-Saint-Isle, France) and kept in the ZMG until the age of 22 months. Rats were housed in standard cages in a climate room with 12 h light and dark phases and free access to food. The American Physiological Society guide principles for the care and use of animals were followed. All animal experiments were approved by the local animal committee (42502-2-1123 MLU, Landesverwaltungsamt Sachsen-Anhalt, Germany). Individuals at the age of 3 months (n = 8), 12 months (n = 8), and 22 months (n = 6) were anaesthetized with 150 mg/kg bodyweight Narcoren (Merial, France). In deep narcosis, animals were killed by exsanguination and tails were removed. Tendons of the same diameter were prepared from the ventral bundles of the skinned tails.

Incubation of Tendons. Tendons at the age of 3 months were incubated separately in 1.5 mL of phosphate buffered saline (PBS, 10 mM phosphate, 150 mM sodium chloride, pH 7.4) containing methylglyoxal (0.2 mM) and diethylenetriaminepentaacetic acid (DTPA, 1 mM) under deaerated conditions at 37 °C in an incubator shaker (New Brunswick Scientific, New Jersey) for 1−7 days. A control was incubated in PBS under the same conditions. Six tendons were incubated for each time point. Afterward, tendons were washed three times with PBS and kept at −20 °C until analyses.

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Table 1. LC–ESI-MS/MS Parameters for AGE Measurements

analyte	retention time [min]	precursor ion		product ion 1[a]			product ion 2[b]			product ion 3[b]		
		m/z	DP[c] [V]	*m/z*	CE[d] [eV]	CXP[e] [V]	*m/z*	CE [eV]	CXP [V]	*m/z*	CE [eV]	CXP [V]
CML	11.6	205.1	42	84.1	30	14	130.2	17	9	56.1	59	9
GALA	12.2	205.2	40	142.1	20	11	84.1	36	14	56.2	64	8
N^6-lactoyl lysine	16.0	219.2	40	156.2	20	8	84.1	35	9	173.1	17	8
CEL	17.1	219.1	54	84.1	33	7	130.1	18	11	56.1	59	8
THP	18.6	319.2	52	70.2	66	11	116.3	36	9	186.4	33	10
G-H3	19.5	215.1	48	100.1	20	8	70.1	38	11	116.2	20	10
CMA	19.8	233.1	55	70.1	43	12	116.1	23	8	118.2	22	6
MG-H3	24.2	229.2	45	114.2	21	9	70.2	45	11	116.1	21	9
CEA	24.4	247.1	51	70.2	48	11	116.2	25	10	132.1	24	10
MG-H1	24.4	229.2	55	166.2	23	12	70.1	43	12	116.1	21	9
LNL	27.4	276.1	70	84.1	44	14	130.1	27	11	213.2	27	16
GOLD	28.6	327.2	60	84.1	51	13	282.3	31	14	198.1	28	14
glucosepane	28.8	429.3	15	384.5	41	19	269.2	55	20	339.2	55	20
GOLA	28.8	333.2	45	84.3	54	13	169.1	26	12	130.2	32	9
DODIC	29.3	447.4	45	301.3	33	16	402.5	42	20	357.2	53	18
MOLD	29.7	341.3	45	296.3	33	18	84.2	52	14	212.3	31	17
MOLA	29.8	347.5	80	84.1	58	7	173.2	31	10	130.2	37	12
GODIC	30.3	343.3	20	298.4	32	7	183.2	44	13	70.2	74	11
argpyrimidine	31.0	255.3	50	70.2	44	12	140.0	24	10	192.1	28	13
MODIC	31.3	357.3	25	197.4	45	14	312.2	35	7	267.3	45	15
pentosidine	31.7	379.2	20	187.3	47	16	316.3	36	8	84.2	59	14

[a]MRM transition used for quantitation (quantifier). [b]MRM transition used for confirmation (qualifier). [c]Declustering potential. [d]Collision energy [e]Cell exit potential.

Tail Tendon Breaking Time Assay. Measurement of tail tendon breaking time (TTBT) of incubated tendons (3 months old) and native tendons of different age (3, 12, and 22 months) was performed as described by Harrison and Archer.[26] Two centimeters of each tendon were fixated with surgical thread at both ends with a slipknot and a weight of 2.746 g. The tendons were immersed in a urea solution (7.0 M urea, 4.3 mM potassium dihydrogen phosphate, 1.4 mM sodium tetraborate, pH 7.5) held at a constant temperature of 40 °C. Time counting was stopped automatically when the tendon broke. TTBT of six tendons per individual were measured for both incubated and native tendons, respectively.

Preparation and Solubilization of Collagen. Tendons were washed with purified water three times and then cut into small pieces. In 2 mL tubes filled with 500 μL of water, tendons were minced with a zirconium oxide grinding ball (5 mm in diameter) in a mixer mill (MM400, Retsch, Germany) at 30 Hz for 30 min. After lyophilization, 3 mg of tendon were solubilized in 1.2 mL of diluted acetic acid (20 mM) at 37 °C and 1000 rpm for 30 min (Thermomixer compact, Eppendorf, Germany) to give a collagen solution.

SDS-PAGE. 100 μL of above collagen solution were predigested with 25 units of pepsin at 37 °C for 1 h. The reaction was stopped by adding 100 μL of SDS solution (160 mM TRIS, 4% (w/v) SDS) and 100 μL of nonreducing Laemmli buffer (125 mM TRIS, 20% (w/v) glycerin, 5% (w/v) SDS, 1 mM EDTA, 0.05% (w/v) bromophenol blue). 20 μL of this solution (20 μg of protein) were analyzed by denaturing polyacrylamide electrophoresis using discontinuous acrylamide gels (resolving gel, T = 6%, C = 3.3%; stacking gel, T = 5%). Protein bands were visualized with colloidal Coomassie staining overnight as described by Dyballa.[27]

Acid Hydrolysis. 400 μL of above collagen solution were evaporated to dryness by vacuum centrifugation (Savant Speed Vac Concentrator, Thermo Scientific, Germany). 200 μL of sodium borohydride (8 mg/mL) in sodium hydroxide (0.01 mM) were added and incubated at room temperature for 1 h. The solution was evaporated to dryness again. After addition of 800 μL of hydrochloric acid (6.0 M), the head space was purged with argon and the reaction tube was sealed and kept in a drying oven at 110 °C for 20 h. Hydrochloric acid was removed by vacuum centrifugation. The dry residue was taken up in 492 μL of diluted hydrochloric acid (0.05 mM) and filtered through a centrifugal tube filter (Costar Spin-X, 0.45 μm, cellulose acetate, Corning Inc., New York).

Enzymatic Hydrolysis. To 500 μL of above collagen solution, 25 units of pepsin were added and incubated at 37 °C for 24 h. The solution was evaporated to dryness by vacuum centrifugation. The residue was taken up in 480 μL of TRIS acetate buffer (TA, 100 mM TRIS, 5 mM calcium chloride, pH 7.4). Stepwise, the following proteases were added every 24 h: 10.0 units of collagenase, again 10.0 units of collagenase, 0.3 units of Pronase E, again 0.3 units of Pronase E, 1.0 unit of leucine aminopeptidase (LAP), and 0.95 units of carboxypeptidase Y. The samples were incubated in an incubator shaker at 37 °C. A small crystal of thymol was added with the first digestion step in TA. After the last digestion step, solutions were filtered through a molecular weight cutoff membrane (MWCO 3000 centrifugal filters, VWR International, Germany). Efficiency of the enzymatic hydrolysis for each sample was compared to the acid hydrolysis by LC–MS/MS analysis of the acid-stable modification N^6-carboxyethyl lysine (CEL).

Preparative Fractionation of Enzymatic Hydrolysates. 150 μL of individual enzymatic hydrolysates were pooled for each age group. The pooled samples were separated by high-performance liquid chromatography (HPLC) on an analytical stainless steel column packed with RP-18 material (Vydac Protein & Peptide C18, 250 mm × 4.6 mm, 5 μm, Grace, Maryland). The HPLC system (Jasco, Germany) consisted of a pump (PU-2080 Plus) with a degasser (DG-2080-54) and a quaternary gradient mixer (LG-2080-04), a column oven (Jetstream II) and an autosampler (AS-2057 Plus). The mobile phase used was water (eluent A) and methanol with water (7:3, v/v, eluent B). 1.2 mL/L of heptafluorobutyric acid was added to both eluents (A and B) as ion pair reagent. Analyses were performed at a column temperature of 25 °C using a flow rate of 1.0 mL/min and gradient elution: 2% B (15 min isocratic), to 70% B (35 min), to 100% B (5 min), and hold (10 min). 100 μL of pooled samples were repetitively injected, and eluate from 37 to 52 min was collected. Eight chromatographic runs were performed in total for each group. The

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combined fraction were evaporated by vacuum centrifugation and freeze-dried. The amorphic material was dissolved in 80 μL of water and analyzed by LC−MS/MS.

Ninhydrin Assay. The amino acid content of acid collagen hydrolysates was measured by the ninhydrin method of Smuda et al. with L-leucine as the reference standard.[28] After derivatization, absorption of the diluted samples and a standard curve was determined at 566 nm with a microplate reader (infinite M200, Tecan, Switzerland) using 96-well plates.

HPLC−MS/MS Analysis. The high-performance liquid chromatography (HPLC) system (Jasco, Germany) consisted of a pump (PU-2080 Plus) with a degasser (DG-2080-02) and a quaternary gradient mixer (LG-2080-04), a column oven (Jetstream II), and an autosampler (AS-2057 Plus). Mass spectrometric detection was conducted on an API 4000 QTrap LC−MS/MS system (Applied Biosystems/AB Sciex, Germany) equipped with a turbo ion spray source using electrospray ionization in positive ion mode: ion spray voltage of 2.5 kV, nebulizing gas pressure of 70 psi, heating gas pressure of 80 psi at 650 °C, and curtain gas pressure of 30 psi. Chromatographic separations were performed on a stainless steel column packed with RP-18 material (Xselect HSS T3, 250 mm × 3.0 mm, 5 μm, Waters, Massachusetts) using a flow rate of 0.7 mL/min. The mobile phase used was water (eluent A) and methanol with water (7:3, v/v, eluent B). 1.2 mL/L of heptafluorobutyric acid were added to both eluents (A and B) as ion pair reagent. Analyses were performed at a column temperature of 25 °C using gradient elution: 2% B (2 min) to 14% B (10 min) to 87% B (22 min) to 100% B (0.5 min) and hold (7 min). For mass spectrometric detection, the scheduled multiple-reaction monitoring (sMRM) mode was used, utilizing collision-induced dissociation (CID) of the protonated molecules with compound specific orifice potentials and fragment specific collision energies (Table 1). Appropriate dilutions of the samples were injected dependent on analyte concentration and matrix interference. Quantitation was based on the standard addition method. More precisely, increasing concentrations of authentic reference compounds at factors of 0.5, 1, and 2 times the concentration of the compounds in the sample were added to separate aliquots of the sample. The aliquots were analyzed, and a regression of response versus concentration was used to determine the concentration of the analyte in the sample. Calibration with this method resolves potential matrix interference.

High-Resolution Mass Determination (HR-MS). Positive and negative ion high-resolution ESI mass spectra were obtained from an Orbitrap Elite mass spectrometer (Thermofisher Scientific, Germany) equipped with an HESI electrospray ion source (spray voltage 4 kV; capillary temperature 275 °C, source heater temperature 40 °C; FTMS resolution >30 000). Nitrogen was used as sheath and auxiliary gas. The sample solutions were introduced continuously via a 500 μL Hamilton syringe pump with a flow rate of 5 μL/min. The data were evaluated by the Xcalibur software 2.7 SP1.

Nuclear Magnetic Resonance Spectroscopy (NMR). NMR spectra were recorded on a VXR 400 spectrometer (Varian, California) operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C or on a Unity Inova 500 instrument (Varian, California) operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C, respectively. Tetramethylsilane was used as a reference for calibrating the chemical shift.

Statistical Analysis. Statistical significance between two adjacent groups was examined using Fisher's test (*F*-test) with a probability value of 95%. All significance tests were performed by two-sample Student's *t*-test or Welch's *t*-test, respectively, with a probability value of at least 95%. Limits of detection (LOD, 3 × S/N) and limits of quantitation (LOQ, 10 × S/N) for each analyte were estimated according to signal/noise ratio (S/N) and are given in Table 2.

RESULTS AND DISCUSSION

Synthesis of MOLA. The present paper aimed to investigate the impact of nonenzymatic protein modification on the physical properties of collagen. Therefore, the formation of Maillard endproducts leading to intra- and intermolecular cross-linking was a particular focus. Interestingly, for the MGO-derived cross-link structures MOLD (methylglyoxal lysine dimer) and MODIC (methylglyoxal-derived imidazoline cross-link), there are also homologous compounds GOLD[29] and GODIC[17] derived from glyoxal (GO). Consequently, a counterpart for the GO-amide cross-link GOLA, which was first described by Glomb and Pfahler[19] in 2001, should be two lysine molecules linked by MGO via an amine and an amide bond, respectively. However, this hypothetical structure has not been described in the literature so far, although Thornalley had already suggested the compound as a "putative crosslink" in 1994.[30] We thus synthesized the novel AGE cross-link mainly according to the synthetic route for GOLA by Glomb and Pfahler using a carbodiimide catalyzed coupling strategy of protected lysine derivatives. The final authentic reference material as well as all intermediates were confirmed by one- and two-dimensional NMR experiments (^{1}H, ^{13}C, H,H-COSY, HSQC, HMBC) and by accurate mass determination. In analogy to GOLA, we named the new compound MOLA for methylglyoxal lysine amide. To date, GOLA and MOLA are the only known glycation cross-links with an amide bond.

Table 2. Limits of Detection (LOD) and Limits of Quantitation (LOQ) for AGEs and Lysinonorleucine (LNL) in Tendon Collagen

	[μmol/mol leu-eq]	
analytes	LOD	LOQ
CEL	1.4	3.6
N^{6}-lactoyl lysine	0.12	0.86
MG-H1	10	13
MG-H3	0.14	0.23
CEA	5.2	10.3
THP	10	35
argpyrimidine	0.03	0.06
CML	1.1	3.6
GALA	0.7	2.2
CMA	3.5	7.3
G-H3	5	12
glucosepane	0.21	0.52
DODIC	0.04	0.19
MODIC	0.005	0.016
MOLA	0.10	0.21
LNL	6	10

Preparation and Solubilization of Tail Tendon Collagen. Rat tail tendons are highly hierarchical constructs consisting of almost exclusively type I collagen. A tendon is built of fiber bundles, whereas fibers are composed of many fibrils. A fibril is formed from single collagen molecules, called tropocollagen, which consist of three polypeptide chains forming a triple helix (~3000 amino acids) with short nonhelical ends, called telopeptides. A high content of glycine (33%), proline (12%), alanine (12%) and 4-hydroxyproline (11%) is characteristic for type I collagen and allows very tight packing of helices and stabilization via hydrogen bonds.[31] Final mechanical and chemical stability results from enzymatic cross-linking of collagen molecules. Especially the explicit water insolubility required the development of an optimized protein workup protocol. To achieve solubility, tendons were first cut into small pieces and minced in a mixer mill to disrupt the fibrillar packing. A fluffy and weighable material resulted after lyophilization. As a result, the processed collagen was then soluble in diluted acetic acid (0.1%, pH 3.5, 37 °C, 30 min).

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Importantly, neither collagen nor amide AGEs were hydrolyzed under these conditions. The protein solution was then aliquoted for both acid and enzymatic hydrolyses. For acid hydrolysis, a reduction step with sodium borohydride converted dehydrolysinonorleucine into the stabile lysinonorleucine (LNL) and avoided CML artifact formation from the Amadori product. Enzymatic hydrolysis was carried out for 7 days at 37 °C by adding fresh proteases every 24 h. Pepsin (25 U) was used in the first digestion step to generate smaller-weight soluble peptides. Collagenase was added (20 U) for the next 48 h to further breakup the collagen peptide structure. The clear solution was then subjected to the standard digestion protocol published by our working group.[28] The complete procedure led to an average hydrolysis efficiency of 73%, which is comparable to values obtained for soluble proteins (e.g., bovine serum albumin).

MOLA in ex Vivo Tendon Incubations. After identifying MOLA in a simple model system comprising of N^2-*t*-Boc-lysine and MGO (data not shown), we investigated AGE formation by MGO in an ex vivo system of 3 months old rat tail tendons. Tendons were incubated under physiological, deaerated conditions over a period of 7 days. Formation of MGO-derived AGEs is shown in Figure 1. As expected, the quantitative

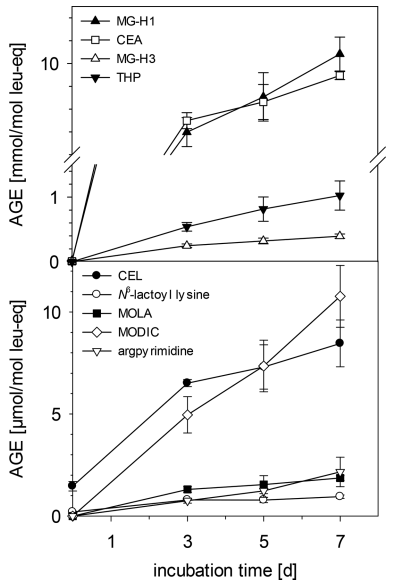

Figure 1. AGE formation in rat tail tendons (3 months) incubated with methylglyoxal (200 μM).

important structures were MG-H1 and N^7-carboxyethyl arginine (CEA). Condensation with the N^5-guanidino function of arginine residues has been reported to be a dominating reaction pathway of MGO.[6] In the first step, MGO condenses with the guanidino group to give the short-lived N^5-endocyclic dihydroxyimidazolidine. After elimination of water, MG-H3 is formed, which is the kinetically controlled product. Hydrolysis converts to the open-chained intermediate CEA to form the thermodynamically controlled N^7-exocyclic MG-H1.[15] This reaction course was reflected by the tendon model. The levels of both MG-H1 and CEA were about 30 times higher than that of MG-H3. THP and to a much lesser extend argpyrimidine are secondary products resulting from the reaction of MG-H3 with another molecule of MGO indicating an excess of MGO in our system. All other structures were found at concentrations 1000 times lower than the hydroimidazolones underlining that arginine guanidino functions are the preferred glycation sites in collagen. In the α_1-chain of collagen type I with a total number of 1052 amino acids, there are 52 arginine and 35 lysine residues[31] explaining the higher capacity of arginine over lysine binding sites. CEL was the dominant lysine modification which is formed nonoxidatively via isomerization from the Schiff base of the keto group of MGO and the N^6-amino function of lysine (Figure 2). Interestingly, in contrast to all other MGO-derived AGEs, native tendons already contained CEL (0 days, 1.5 μmol/mol leucine-equivalents). The lysine-arginine cross-link MODIC was formed at the same concentration levels as CEL until 3 days. After that, CEL formation slowed down, while MODIC formation proceeded to increase linearly. N^6-lactoyl lysine is the amide analogue of CEL formed from the Schiff base adduct of the aldehyde group of MGO. Thus, formation of CEL and N^6-lactoyl lysine is competitive and driven by the reactivity and availability of the respective carbonyl moiety. In our experiment, CEL levels were constantly about 10 times higher. This ratio can be explained by the initial step of Schiff base formation. In line with the carbonyl reactivity, free MGO (<1%) is in equilibrium with its mono- (56%) and dihydrate (44%) in aqueous solution.[32] Thus, the monohydrate form showing a free keto group is much more available for nucleophilic attack than the aldehyde group of free MGO. Based on the isomerization mechanism leading to N^6-lactoyl lysine, we postulate the formation of MOLA. After formation of the hemiaminal at the aldehyde group, a second lysine residue attacks the free keto group and rearrangement leads to MOLA. Indeed, MOLA formation proceeded in parallel to that of CEL and N^6-lactoyl lysine in our system. MOLA levels were twice as high as N^6-lactoyl lysine levels. In accordance with above overall amino acid content, arginine modification increased in a linear fashion indicating still free reaction sites while lysine modification slowed down after 3 days indicating limitations of formation, e.g., de novo synthesis of MODIC and CEL was almost the same until 3 days but diverted to 11 and 8 μmol/mol leucine-equivalents after 7 days, respectively. Comparison of MODIC and MOLA formation suggested that cross-linking in collagen is especially favored between lysine and arginine side chains in collagen due to sterical aspects. Gautieri et al. simulated possible cross-linking sites in collagen fibrils in silico and indeed identified 14 possible lysine-arginine pairs of which six were intra- and eight were intermolecular.[33]

Mechanical Properties of Incubated and Aged Tendons. To evaluate the effect of MGO-mediated glycation on the physical characteristics of collagen, one part of each incubated tendon was subjected to the tail tendon breaking time (TTBT) assay as described by Harrison and Archer.[26] Accordingly, TTBT mainly depends on covalent cross-linking within the collagen fibrils. The results are shown in Figure 3 for the present tendon incubations. Increase of TTBT from 10 min for native tendons to 600 min after 7 days correlated with increasing AGE levels, especially of the cross-link structures MODIC and MOLA. Several prerequisites were chosen to ensure that ex vivo incubation conditions were at least related to conditions found during the natural aging process. Preliminary experiments confirmed that MGO-glycation cross-links are formed nonoxidatively. Furthermore, reactive oxygen species like hydrogen peroxide lead to collagen cross-linking even in absence of glycation agents.[4] For that reason, all incubation solutions were purged with helium to provide

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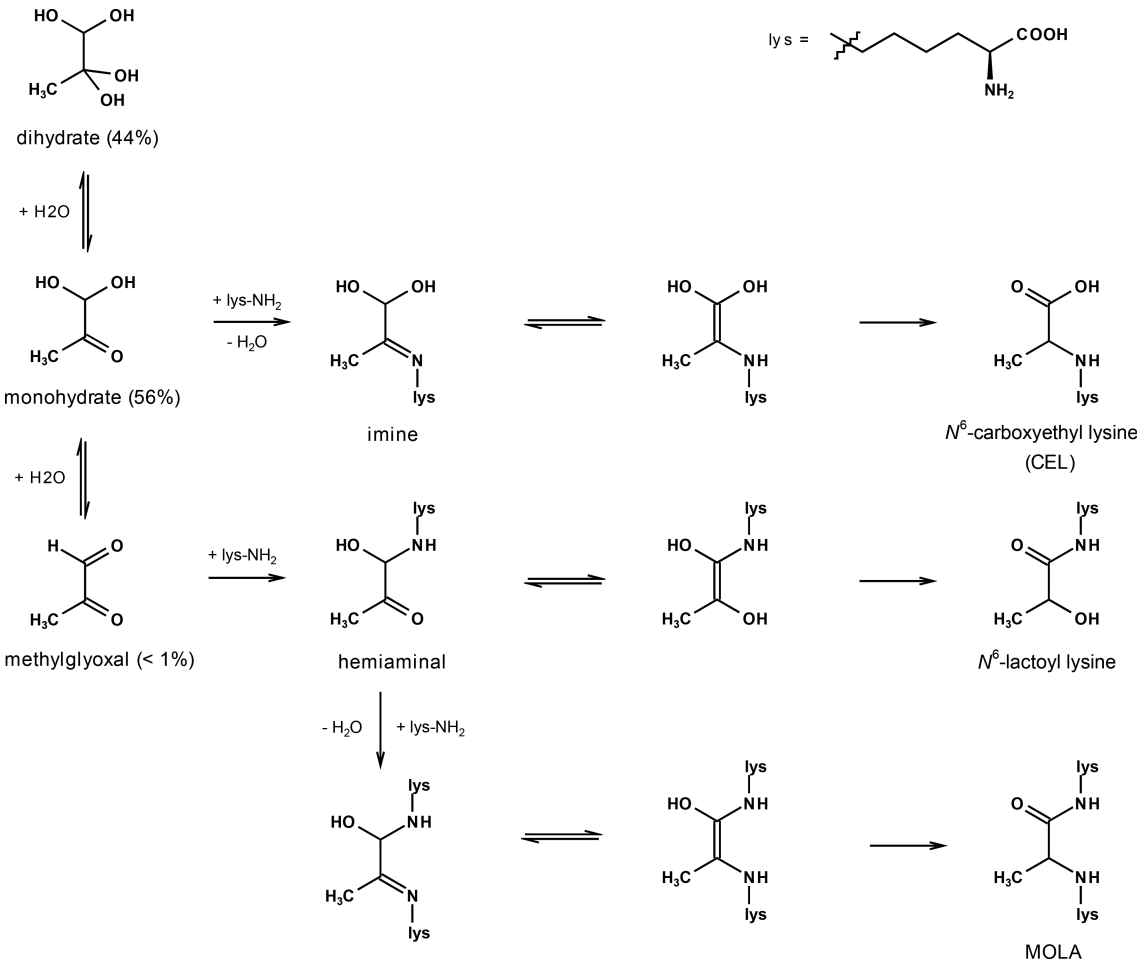

Figure 2. Postulated mechanism for the formation of MOLA according to the CEL and N^6-lactoyl lysine reaction cascade.[1]

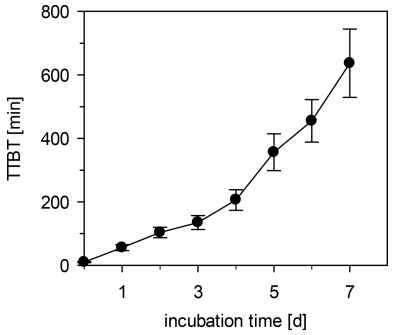

Figure 3. Tail tendon breaking time of rat tail tendons (3 months) incubated with methylglyoxal (200 μM).

strictly deaerated conditions. Second, only freshly prepared MGO was used to avoid side reactions of contaminants in commercial MGO solutions like formaldehyde.[15] Third, compared to literature a relatively low MGO concentration of 200 μM was chosen.[6] In relation to MGO levels of 2 μM normally found in rat plasma, this is still very high but allowed to monitor TTBTs comparable to those found in vivo within the given incubation time frame. Test incubations with MGO concentrations of 1 mM and 10 mM resulted in extremely long or not measurable TTBTs. From a mechanistic point of view, high dicarbonyl levels will shift the AGE spectrum to structures that are unlikely to form under physiological conditions which are characterized by low carbonyl but high amine concentrations.[17,18] In the case of MGO for arginine modifications, high amounts of THP and argpyrimidine and for lysine modifications specifically the cross-link structure MOLD would result. In the present ex vivo setup at 200 μM MOLD was never detected. In parallel to the TTBT assay, gel electrophoresis of the glycated tendons was conducted (Figure 4). After predigestion with pepsin for 1 h, soluble polypeptide cleavage

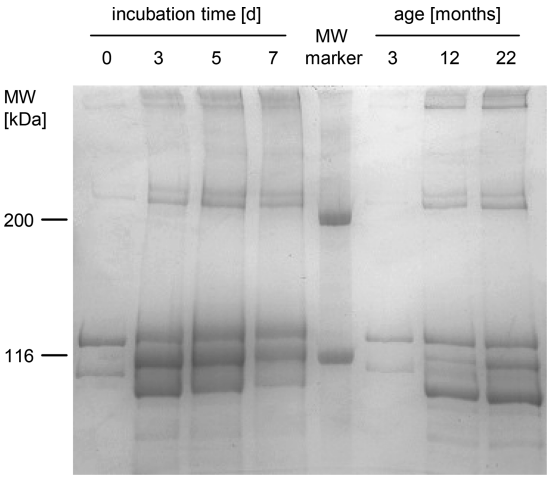

Figure 4. SDS-PAGE of rat tail tendons (3 months) incubated with methylglyoxal (200 μM) and native rat tail tendons of different ages.

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fragments with a molecular weight of around 116 kDa and over 200 kDa increased with incubation time up to 5 days indicating a progress of covalent cross-linking. At 7 days these bands faded out, as the highly glycated collagen became less digestible and major portions could no longer enter the gel.

The TTBT assay was then performed on native tendons of different ages (3, 12, and 22 months, Figure 5). TTBT

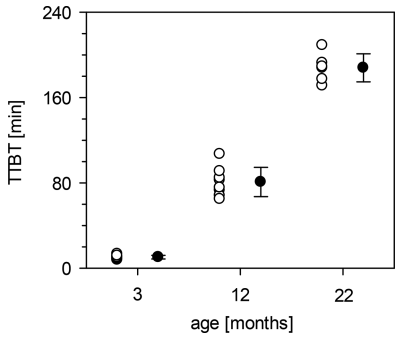

Figure 5. Tail tendon breaking time of native rat tail tendons of different age.

increased from 10 min of young, to 80 min for adult, to 190 min for old individuals (r = 0.986, $P \leq 0.001$). These findings clearly confirmed that cross-linking proceeds with age. However, in vivo enzymatic cross-linking of collagen occurs in parallel to nonenzymatic processes. In immature collagen, lysyl oxidase (LOX)-mediated cross-links are labile aldimines, ketoamines, or aldol condensation products. During maturation of collagen, these bivalent structures are slowly converted into tri- and tetravalent cross-links between the collagen fibrils.[2] Interestingly, the total number of enzymatic cross-links remains constant over time due to a decreased LOX activity and a limited number of lysine residues in the telopeptide region.[34] As a probe for these processes, we determined lysinonorleucine (LNL) after reduction in acidic hydrolysates. LNL is the reduced form of the immature aldimine cross-link dehydrolysinonorleucine. In compliance to literature, LNL decreased with age from 32 to 20 μmol/mol leucine-equivalents (Table 3). In contrast to LOX-modified lysine residues located in the telopeptides, lysine side chains within the triple helix remain susceptible to glycation during aging leading to accumulation of nonenzymatic cross-link structures.[2] Consequently, AGE cross-links will significantly contribute to the increased TTBT in aged tendons. SDS-PAGE of aged tendons revealed a similar cross-linking pattern as in MGO-glycated tendons (Figure 4), however, to a much lesser extent.

Quantitation of MOLA and Other AGEs in Vivo. To elucidate AGE candidates responsible for collagen cross-linking during aging, native rat tendons of different age were processed via the optimized workup protocol. Results of AGE quantitation are summarized in Table 3. In line with above ex vivo MGO-incubation, MG-H1 was the major modification in young, adult, and old tail tendon collagen. Similar findings were made in different extracellular matrix proteins.[35] In our study, MG-H1, CEA, and MG-H3 increased significantly with age totaling up to 34 μmol/mol leucine-equivalents. The ratio between these structures reflected again the above-described mechanistic relationship as in the ex vivo incubations. Although CEA and the hydroimidazolones are no cross-linking structures, monovalent modification of arginine residues will change the isoelectric point of collagen molecules affecting physical

Table 3. AGE and Lysinonorleucine (LNL) Levels[a] in Native Rat Tail Tendon Collagen of Different Age

	age [months]		
analyte [μmol/mol leu-eq]	3	12	22
MGO-Derived			
CEL	1.5 ± 0.2	1.6 ± 0.2	1.8 ± 0.1*
N^6-lactoyl lysine	0.20 ± 0.03	0.32 ± 0.07**	0.5 ± 0.1*
MG-H1	19 ± 1	21 ± 2**	25 ± 1***
MG-H3	0.57 ± 0.04	0.65 ± 0.04**	0.75 ± 0.03***
CEA	7.2 ± 0.6	7.4 ± 0.5	8.0 ± 0.3*
GO-Derived			
CML	4.7 ± 0.5	6.9 ± 0.7**	13 ± 1**
GALA	0.6 ± 0.1	0.7 ± 0.1	1.1 ± 0.4*
CMA	3.1 ± 0.5	6 ± 1***	8 ± 1*
G-H3[b]	21 ± 2	23 ± 2	21 ± 2
Glycation Cross-Links[c]			
glucosepane	n. d.	<LOQ	0.72
DODIC	n. d.	<LOQ	0.24
MODIC	n. d.	<LOQ	0.04
MOLA	n. d.	<LOQ	0.34
Enzymatic Cross-Link[d]			
LNL	32 ± 3	22 ± 1**	20 ± 2*

[a]AGE levels are given as mean values ± standard deviation; statistical significance between adjacent groups: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. [b]Sum parameter for dihydroxyimidazolidine and carboxymethylarginine (CMA) after acid hydrolysis. [c]After preparative enrichment (n. d., not detectable). [d]After reduction and acid hydrolysis.

properties like molecular packing but also biological function.[34] The MGO-lysine modifications CEL and N^6-lactoyl lysine were also identified in tendon collagen at lower concentrations up to 2.3 μmol/mol leucine-equivalent with a low but still significant increase in old tendons. Unlike to ex vivo experiments, a wide array of other AGEs was detected due to the occurrence of many additional 1,2-dicarbonyls in vivo.[36] A major group were GO-derived lysine and arginine modifications. CML was the most prominent lysine modification reaching 13 μmol/mol leucine-equivalents due to its multiple formation pathways.[1] In addition to GO-isomerization reactions, the predominant pathway in vivo is the oxidative fragmentation of fructosyl lysine. In contrary, GALA is a GO-specific alternative lysine modification from the CML isomerization cascade. GALA was about 10 times lower than CML which is not according to the ratio found for the pendants N^6-lactoyl lysine and CEL. CML levels were 7-fold higher than CEL in old individuals. This again reflects that major portions of CML must originate from other sources, specifically the Amadori product. N^7-Carboxymethyl arginine (CMA) is a GO-specific arginine modification.[21] In acidic tendon hydrolysates, imidazolinone G-H3 was quantitated to estimate the total amount of arginine-bound GO. G-H3 is formed from both CMA and the dihydroxyimidazolidine under the harshly acidic conditions used for total protein hydrolysis.[21] Thus, G-H3 can be regarded as a sum parameter for both structures. Interestingly, CMA increased significantly with age while G-H3 remained unchanged. This suggests that (I) most glyoxal is bound as dihydroxyimidazolidine at young age and converts slowly into CMA over time and (II) that total

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GO-arginine modification does not accumulate in collagen with age.

In the original workup of both acidic and enzymatic hydrolysates no AGE cross-link structures were identified. For that reason, enzymatic hydrolysates of each age group were pooled and repetitively fractionated by analytical HPLC on a quantitative basis. The 10-fold concentrated samples were analyzed again and four glycation cross-links were quantitated. Glucosepane was the dominant structure in old tendons with 0.72 μmol/mol leucine-equivalents which is in support of the literature.[5,8] Considering the amino acid composition of type I collagen, this can be estimated as one glucosepane molecule per 600 collagen molecules. This lysine-arginine cross-link is formed from the Lederer's glucosone resulting from degradation of the Amadori product. Amounts of MOLA and DODIC were about half and one-third of glucosepane concentrations, respectively. Surprisingly, MODIC, which was the dominant structure in the ex vivo incubations, was only formed in traces. These findings indicate in line with the above-discussed conformational conditions in type I collagen that covalent cross-linking of lysine and arginine residues was obviously favored over lysine−lysine cross-linking. Second, cross-linking was mainly mediated by the Amadori product and its dicarbonyl follow-up structures Lederer's glucosone and 3-deoxyglucosone. Nevertheless, MOLA was the only quantitatively relevant lysine−lysine cross-link derived from MGO found in old collagen. With a concentration of 0.34 μmol/mol leucine-equivalents this calculates to approximately one MOLA molecule per 1200 collagen molecules. In comparison, levels found for CEL and MG-H1 correspond to one molecule per 250 and 20 collagen molecules, respectively. Other AGE cross-links, more precisely GOLA, GOLD, GODIC, MOLD, and pentosidine were also analyzed but were not detected.

In conclusion, collagen is proceedingly modified by glycation during aging. For the first time, based on an optimized protein workup protocol rat tail tendons were comprehensively analyzed by LC−MS/MS for monovalent and cross-link modifications. MGO was verified as the major glycating agent leading up to 36 μmol/mol leucine-equivalents of mainly arginine directed monovalent modifications. MOLA, a novel lysine−lysine amide cross-link derived from methylglyoxal, was established for the first time as a quantitatively relevant cross-linking structure in senescent collagen. Chemical cross-linking was also visualized by SDS-PAGE and correlated with increase in collagen stiffening measured as TTBT.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

AGE, advanced glycation endproduct; CEA, N^7-carboxyethyl arginine; CEL, N^6-carboxyethyl lysine; CMA, N^7-carboxymethyl arginine; CML, N^6-carboxymethyl lysine; DODIC, 3-deoxyglucosone-derived imidazoline cross-link; GALA, N^6-glycoloyl lysine (glycolic acid lysine amide); G-H3, glyoxal hydroimidazolone; GODIC, glyoxal-derived imidazoline cross-link; GOLA, glyoxal lysine amide; GOLD, glyoxal lysine dimer; leu-eq, leucine-equivalents; LNL, lysinonorleucine; MG-H1/3, methylglyoxal hydroimidazolone 1/3; MGO, methylglyoxal; MODIC, methylglyoxal-derived imidazoline cross-link; MOLA, methylglyoxal lysine amide; MOLD, methylglyoxal lysine dimer; MW, molecular weight; n. d., not detectable; PBS, phosphate buffered saline; THP, tetrahydropyrimidine; TTBT, tail tendon breaking time

REFERENCES

(1) Henning, C.; Glomb, M. A. Pathways of the Maillard Reaction under Physiological Conditions. *Glycoconjugate J.* **2016**, *33*, 499−512.

(2) Bailey, A. Molecular Mechanisms of Ageing in Connective Tissues. *Mech. Ageing Dev.* **2001**, *122*, 735−755.

(3) Gelse, K. Collagens - Structure, Function and Biosynthesis. *Adv. Drug Delivery Rev.* **2003**, *55*, 1531−1546.

(4) Monnier, V. M.; Glomb, M.; Elgawish, A.; Sell, D. R. The Mechanism of Collagen Crosslinking in Diabetes. A Puzzle Nearing Resolution,. *Diabetes* **1996**, *45*, S67−S72.

(5) Monnier, V. M.; Mustata, G. T.; Biemel, K. L.; Reihl, O.; Lederer, M. O.; Zhenyu, D.; Sell, D. R. Crosslinking of the Extracellular Matrix by the Maillard Reaction in Aging and Diabetes. An Update on "A Puzzle Nearing Resolution",. *Ann. N. Y. Acad. Sci.* **2005**, *1043*, 533−544.

(6) Fessel, G.; Li, Y.; Diederich, V.; Guizar-Sicairos, M.; Schneider, P.; Sell, D. R.; Monnier, V. M.; Snedeker, J. G.; Screen; Hazel, R. C. Advanced Glycation Endproducts Reduce Collagen Molecular Sliding to Affect Collagen Fibril Damage Mechanisms but Not Stiffness. *PLoS One* **2014**, *9*, e110948.

(7) Skovgaard, D.; Svensson, R. B.; Scheijen, J.; Eliasson, P.; Mogensen, P.; Hag, A. M. F.; Kjaer, M.; Schalkwijk, C. G.; Schjerling, P.; Magnusson, S. P.; Couppe, C. An Advanced Glycation Endproduct (AGE)-Rich Diet Promotes Accumulation of AGEs in Achilles Tendon. *Physiol. Rep.* **2017**, *5*, e13215.

(8) Sell, D. R.; Biemel, K. M.; Reihl, O.; Lederer, M. O.; Strauch, C. M.; Monnier, V. M. Glucosepane is a Major Protein Crosslink of the Senescent Human Extracellular Matrix. *J. Biol. Chem.* **2005**, *280*, 12310−12315.

(9) Thornalley, P. J. Pharmacology of Methylglyoxal: Formation, Modification of Proteins and Nucleic Acids, and Enzymatic Detoxification. *Gen. Pharmacol.* **1996**, *27*, 565−573.

(10) Kalapos, M. P. Where does plasma methylglyoxal originate from? *Diabetes Res. Clin. Pract.* **2013**, *99*, 260−271.

(11) Nagaraj, R. H.; Sarkar, P.; Mally, A.; Biemel, K. M.; Lederer, M. O.; Padayatti, P. S. Effect of pyridoxamine on chemical modification of proteins by carbonyls in diabetic rats: characterization of a major product from the reaction of pyridoxamine and methylglyoxal. *Arch. Biochem. Biophys.* **2002**, *402*, 110−119.

(12) Rabbani, N.; Thornalley, P. J. Methylglyoxal, glyoxalase 1 and the dicarbonyl proteome. *Amino Acids* **2012**, *42*, 1133−1142.

DOI: 10.1021/acs.jafc.8b00937
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

(13) Ahmed, M. U.; Frye, E. B.; Degenhardt, T. P.; Thorpe, S. R.; Baynes, J. W. N^{ε}-Carboxyethyl Lysine, a Product of the Chemical Modification of Proteins by Methylglyoxal, Increases with Age in Human Lens Proteins. *Biochem. J.* **1997**, *324*, 565−570.
(14) Smuda, M.; Voigt, M.; Glomb, M. A. Degradation of 1-deoxy-D-erythro-hexo-2,3-diulose in the presence of lysine leads to formation of carboxylic acid amides. *J. Agric. Food Chem.* **2010**, *58*, 6458−6464.
(15) Klöpfer, A.; Spanneberg, R.; Glomb, M. A. Formation of Arginine Modifications in a Model System of N^{α}-*tert*-Butoxycarbonyl (Boc)-Arginine with Methylglyoxal. *J. Agric. Food Chem.* **2011**, *59*, 394−401.
(16) Nagaraj, R. H.; Shipanova, I. N.; Faust, F. M. Protein Crosslinking by the Maillard Reaction: Isolation, Characterization and *in vivo* Detection of a Lysine-Lysine Crosslink Derived from Methylglyoxal. *J. Biol. Chem.* **1996**, *271*, 19338−19345.
(17) Lederer, M. O.; Klaiber, R. G. Crosslinking of Proteins by Maillard Processes. Characterization and Detection of Lysine-Arginine Crosslinks Derived from Glyoxal and Methylglyoxal. *Bioorg. Med. Chem.* **1999**, *7*, 2499−2507.
(18) Glomb, M. A.; Monnier, V. M. Mechanism of Protein Modification by Glyoxal and Glycolaldehyde, Reactive Intermediates of the Maillard Reaction. *J. Biol. Chem.* **1995**, *270*, 10017−10026.
(19) Glomb, M. A.; Pfahler, C. Amides Are Novel Protein Modifications Formed by Physiological Sugars. *J. Biol. Chem.* **2001**, *276*, 41638−41647.
(20) Odani, H.; Iijima, K.; Nakata, M.; Miyata, S.; Yasuda, Y.; Irie, S.; Maeda, K.; Fujimoto, D. Identification of N^{ω}-Carboxymethyl Arginine, as a new Advanced Glycation Endproduct in Serum Proteins of Diabetic Patients. *Int. Congr. Ser.* **2002**, *1245*, 295−301.
(21) Glomb, M. A.; Lang, G. Isolation and Characterization of Glyoxal−Arginine Modifications. *J. Agric. Food Chem.* **2001**, *49*, 1493−1501.
(22) Shipanova, I. N.; Glomb, M. A.; Nagaraj, R. H. Protein Modification by Methylglyoxal. Chemical Nature and Synthetic Mechanism of a Major Fluorescent Adduct. *Arch. Biochem. Biophys.* **1997**, *344*, 29−36.
(23) Lederer, M. O.; Bühler, H. P. Crosslinking of Proteins by Maillard Processes. Characterization and Detection of a Lysine-Arginine Crosslink Derived from D-Glucose. *Bioorg. Med. Chem.* **1999**, *7*, 1081−1088.
(24) Biemel, K. M.; Reihl, O.; Conrad, J.; Lederer, M. O. Formation Pathways for Lysine-Arginine Crosslinks Derived from Hexoses and Pentoses by Maillard Processes. Unraveling the Structure of a Pentosidine Precursor. *J. Biol. Chem.* **2001**, *276*, 23405−23412.
(25) Strazzolini, P.; Melloni, T.; Giumanini, A. G. Selective Nitrolytic Deprotection of N-Boc-Amines and N-Boc-Amino Acids Derivatives. *Tetrahedron* **2001**, *57*, 9033−9043.
(26) Harrison, D. E.; Archer, J. R. Measurement of Changes in Mouse Tail Collagen with Age. Temperature dependence and procedural details. *Exp. Gerontol.* **1978**, *13*, 75−82.
(27) Dyballa, N.; Metzger, S. Fast and Sensitive Colloidal Coomassie G-250 Staining for Proteins in Polyacrylamide Gels. *J. Visualized Exp.* **2009**, DOI: 10.3791/1431.
(28) Smuda, M.; Henning, C.; Raghavan, C. T.; Johar, K.; Vasavada, A. R.; Nagaraj, R. H.; Glomb, M. A. Comprehensive Analysis of Maillard Protein Modifications in Human Lenses: Effect of Age and Cataract. *Biochemistry* **2015**, *54*, 2500−2507.
(29) Chellan, P.; Nagaraj, R. H. Protein Crosslinking by the Maillard Reaction and diabetes. Dicarbonyl-derived Imidazolium Crosslinks in Aging. *Arch. Biochem. Biophys.* **1999**, *368*, 98−104.
(30) Thornalley, P. J.; Westwood, M. E.; McLellan, A. C.; Lo, T. W. Binding and Modification of Proteins by Methylglyoxal under Physiological Conditions. *J. Biol. Chem.* **1994**, *51*, 32299−32305.
(31) Hulmes, D. J.; Miller, A.; Parry, D. A.; Piez, K. A.; Woodhead-Galloway, J. Analysis of the primary structure of collagen for the origins of molecular packing. *J. Mol. Biol.* **1973**, *79*, 137−148.
(32) McLellan, A. C.; Thornalley, P. J. Synthesis and Chromatography of 1,2-Diamino-4,5-dimethoxybenzene, 6,7-Dimethoxy-2-methylquinoxaline and 6,7-Dimethoxy-2,3-dimethylquinoxaline for Use in a Liquid Chromatographic Fluorimetric Assay of Methylglyoxal. *Anal. Chim. Acta* **1992**, *263*, 137−142.
(33) Gautieri, A.; Redaelli, A.; Buehler, M. J.; Vesentini, S. Age- and Diabetes-related Non-enzymatic Crosslinks in Collagen Fibrils: Candidate Amino Acids Involved in Advanced Glycation Endproducts. *Matrix Biol.* **2014**, *34*, 89−95.
(34) Avery, N. C.; Bailey, A. J. The Effects of the Maillard Reaction on the Physical Properties and Cell Interactions of Collagen,. *Pathol. Biol.* **2006**, *54*, 387−395.
(35) Ahmed, N.; Thornalley, P. J.; Dawczynski, J.; Franke, S.; Strobel, J.; Stein, G.; Haik, G. M. Methylglyoxal-Derived Hydroimidazolone Advanced Glycation End-Products of Human Lens Proteins, *Invest. Ophthalmol. Invest. Ophthalmol. Visual Sci.* **2003**, *44*, 5287−5292.
(36) Henning, C.; Liehr, K.; Girndt, M.; Ulrich, C.; Glomb, M. A. Extending the Spectrum of α-Dicarbonyl Compounds *in vivo*. *J. Biol. Chem.* **2014**, *289*, 28676−28688.

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