Molecular Basis of Maillard Amide-Advanced Glycation End Product (AGE) Formation in Vivo

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The Maillard reaction in vivo entails alteration of proteins or free amino acids by non-enzymatic glycation or glycoxidation. The resulting modifications are called advanced glycation end products (AGEs) and play a prominent role in various pathologies, including normoglycemic uremia. Recently, we established a new class of lysine amide modifications was established in vivo.

Background: Advanced glycation end products (AGEs) play a prominent role in various pathologies.

Results: A new class of lysine amide modifications was established in vivo.

Conclusion: Non-enzymatic Maillard mechanisms participate on amide-AGE formation pathways in vivo.

Significance: Plasma levels of the amide-AGEs were in the same range similar to other established lysine modifications and suggest a comparable impact of non-enzymatic biochemistry on pathophysiology in the human organism.

The Maillard reaction in vivo entails alteration of proteins or free amino acids by non-enzymatic glycation or glycoxidation. The resulting modifications are called advanced glycation end products (AGEs) and play a prominent role in various pathologies, including normoglycemic uremia. Recently, we established a new class of lysine amide modifications in vivo. Now, human plasma levels of the novel amide-AGEs N6-acetyl lysine, N6-formyl lysine, N6-lactoyl lysine, and N6-glycerinyl lysine were determined by means of LC-MS/MS. They were significantly higher in uremic patients undergoing hemodialysis than in healthy subjects. Model reactions with N6-t-butyloxycarbonyllysine under physiological conditions confirmed 1-deoxy-D-erythro-hexo-2,3-diulose as an immediate precursor. Because formation of N6-formyl lysine from glucose responded considerably to the presence of oxygen, glucosone was identified as another precursor. Comparison of the in vivo results with the model experiments enabled us to elucidate possible formation pathways linked to Maillard chemistry. The results strongly suggest a major participation of non-enzymatic Maillard mechanisms on amide-AGE formation pathways in vivo, which, in the case of N6-acetyl lysine, parallels enzymatic processes.
strain mice, which naturally develop diabetes with age. The lysine adduct was discussed as one of several potential biomarker candidates related to diabetes mellitus.

Nε-formyl lysine is formed in vitro by reaction of albumin with trichloroethylene oxide, a major metabolite of 1,1,2-trichloroethylene, one of the most common compounds found in chemical waste dumps (21). In addition, it could be identified in incubation mixtures of various sugars, l-ascorbic acid or l-dehydroascorbic acid with poly-l-lysine and β-lactoglobulin, respectively (22). Jiang et al. (23) observed transfer of formyl groups from 3'-formylphosphate-ended DNA, arising from oxidation of the 5’-position of deoxyribose and subsequent DNA strand breakage by the endonuclease antibiotic neocarzinostatin, to histone proteins in human TK6 cells to give Nε-formyl lysine.

The aim of the present work was to elucidate formation and relevance of the four amide-AGEs Nε-acetyl lysine, Nε-formyl lysine, Nε-lactoyl lysine, and Nε-glycerinyl lysine (structures are shown in Fig. 5) in vivo with regard to non-enzymatic mechanisms within the Maillard reaction. Therefore, the free adducts were analyzed in human blood plasma by means of an LC-MS/MS technique. In model experiments, formation from glucose, the physiologically most important sugar, and from various α-dicarbonyl structures as direct precursors was confirmed. Comparison of in vivo with in vitro results allowed us to assess the impact of Maillard chemistry on amide-AGE formation in vivo.

**EXPERIMENTAL PROCEDURES**

**Plasma Samples**—Written informed consent was obtained from all patients. The study was approved by the Ethics Committee of the Medical Faculty of the Martin Luther University Halle-Wittenberg. Blood samples were obtained from 11 healthy subjects (controls) with normal renal function and 18 non-diabetic patients undergoing hemodialysis (HD patients) with trichloroethylene oxide, a major metabolite of 1,1,2-trichloroethylene, one of the most common compounds found in chemical waste dumps (21). In addition, it could be identified in incubation mixtures of various sugars, l-ascorbic acid or l-dehydroascorbic acid with poly-l-lysine and β-lactoglobulin, respectively (22). Jiang et al. (23) observed transfer of formyl groups from 3'-formylphosphate-ended DNA, arising from oxidation of the 5’-position of deoxyribose and subsequent DNA strand breakage by the endonuclease antibiotic neocarzinostatin, to histone proteins in human TK6 cells to give Nε-formyl lysine.

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**Materials**—Chemicals of the highest grade available were obtained from Sigma-Aldrich and Thermo Fisher Scientific unless otherwise indicated. 1-deoxy-1-(Nε-lysino)-D-fructose (Amadori product) (24), 3-DG (25), β-arabino-hexos-2-ulos (glucose) (26), 1-DG and 2-ethyl-3-methylquinoline (27), CML (28), Nε-carboxyethyl lysine (CEL) (29), and Nε-lactoyl lysine and Nε-glycerinyl lysine (7) were synthesized according to the literature. The identities of target compounds were verified by nuclear magnetic resonance experiments. Furthermore, the elemental composition was confirmed by accurate mass determination.

**Model Reactions**—In general, incubations were conducted in 0.1 M phosphate buffer, pH 7.4, after sterile filtration in a shaker incubator (New Brunswick Scientific, Nürtinngen, Germany) at 37 °C. The reactant concentrations are mentioned in the legend to the corresponding figures and tables. Deaeration and inhibition of metal-catalyzed oxidation chemistry was achieved by using phosphate buffer containing 1 mM diethylenetriaminepentaacetic acid and gassing with argon. Buffer was degassed with helium and stored under argon prior to sample preparation. At various time points aliquots of the reaction mixtures were diluted with 6 M HCl to a final HCl concentration of 3 M. For quantitative removal of the Boc protection group, the samples were kept at room temperature for 30 min. After dilution to appropriate concentrations, the solutions were subjected to LC-MS/MS analysis.

**Assay of Amide-AGEs, CML, and CEL in Plasma**—200 μl of plasma was transferred into a centrifugal filter containing a modified polyethersulfone membrane with 3 kDa molecular weight cut-off (VWR International, Darmstadt, Germany). Centrifugation was carried out at 14,000 × g and 4 °C. The filtrate was diluted on a scale of 1:20 with 0.1% HCl and administered to LC-MS/MS analysis.

**Assay of Methylglyoxal in Plasma**—Methylglyoxal in plasma samples was analyzed according to a modified method by McLellan et al. (8). Briefly, 500 μl of plasma were incubated...
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with o-phenylenediamine at room temperature in the dark for 24 h under acidic conditions. 2-Ethyl-3-methylquinoxaline was used as internal standard. The filtered supernatant was subjected to LC-MS/MS analysis.

High Performance LC-MS/MS—The HPLC apparatus (Jasco, Gross-Umstadt, Germany) consisted of a pump (PU-2080 Plus) with degasser (LG-2080-02) and quaternary gradient mixer (LG-2080-04), a column oven (Jasco Jetstream II), and an autosampler (AS-2057 Plus). Mass spectrometric detection was conducted on an API 4000 QTrap LC-MS/MS system (Applied Biosystems/MDS Sciex, Concord, ON, Canada) equipped with a turbo ionspray source using electrospray ionization in positive mode: sprayer capillary voltage, 4.0 kV; nebulizing gas flow, 1.0 ml/min; heating gas, 60 ml/min at 550 °C; and curtain gas, 40 ml/min.

For the detection of the amide-AGEs, CML and CEL, chromatographic separations were performed on a stainless steel column packed with RP-18 material (Vydac CRT, no. 218TP54, 250 × 4.0 mm, RP-18, 5 μm, Hesperia, CA) using a flow rate of 1.0 ml/min. The mobile phase used was water (solvent A) and methanol/water (7:3 (v/v), solvent B). To both solvents (A and B), 1.2 ml/liter heptafluorobutyric acid was added. Analysis was performed at 35 °C column temperature using isocratic elution at 98% A/2% B. For mass spectrometric detection, the multiple reaction monitoring mode was used, utilizing collision-induced dissociation of the protonated molecules with compound specific orifice potentials and fragment-specific collision energies (Table 1).

Quantification was performed using the standard addition method. More precisely, increasing concentrations of authentic reference compounds at factors of 0.5, 1, 2, and 3 × the concentration of the analyte in the sample were added to separate aliquots of the sample after workup procedure. The aliquots were analyzed, and a regression of response versus concentration was used to determine the concentration of the analyte in the sample. Spikes were run one in approximately every 30 samples. Calibration with this method resolves potential matrix interferences.

To obtain fragmentation spectra of amide-AGEs in plasma workup solutions, target material was first enriched by repeated collection from the above HPLC system. After solvent evaporation in a vacuum concentrator (Savant SpeedVac Plus SC 110 A combined with a Vacuum Trap RVT 400, Thermo Fisher Scientific GmbH), the residue was dissolved in water and reinfected, using a collision-induced dissociation experiment. The fragmentation spectra of the authentic references were obtained with the same parameters. For N²-lactoyl lysine, the following parameters were used: declustering potential (DP), 33 V; collision energy (CE), 23 eV; collision cell exit potential (CXP), 8 V; and scan range (m/z), 50–220 (2 s).

For the detection of methylglyoxal quinoxaline, chromatographic separations were performed on a stainless steel column (Knauer, Eurospher 100 C18, 5 μm, 250 × 4.6 mm, Berlin, Germany) using a flow rate of 1.0 ml/min. The mobile phase used was water (solvent A) and methanol/water (7:3 (v/v), solvent B). To both solvents (A and B), 0.6 ml/liter heptafluorobutyric acid was added. Analysis was performed at 35 °C column temperature using gradient elution: 20 (0) to 30 (35) to 100 (65–70) to 20 (75–85); % B (t/min). For mass spectrometric detection, the multiple reaction monitoring mode was used, utilizing a collision-induced dissociation of the protonated molecules with MS parameters as follows: m/z 229.2 → 77.0 (DP, 50 V; CE, 41.0 eV; CXP, 5.0 V, quantifier), m/z 229.2 → 118.1 (DP, 50 V; CE, 30.5 eV; CXP, 7.0 V; qualifier), m/z 229.2 → 65.0 (DP, 50 V; CE, 45.0 eV; CXP, 5.0 V, qualifier). Methylglyoxal quinoxaline was detected at the retention time of tR = 58.1 min. Quantification was performed using the standard addition method with pure authentic reference compounds.

All plasma workup samples and incubations were analyzed in single batches to exclude interassay variations. Intra-assay coefficients of variation were determined by repeated analyses of controls and HD patients. In addition, as shown in Table 2, limit of detection (LOD) and limit of quantification (LOQ) with all steps of the analysis included were estimated according to DIN 32645 (n = 6, confidence level, p = 0.95, k = 3) (30). In model experiments, coefficients of variation < 10% (n = 3) for all target compounds was achieved, and LOD/LOQ was 0.0004/0.001 mmol/mol lysine for acetyl lysine, 0.0013/0.004 mmol/mol lysine for formyl lysine, 0.0007/0.002 mmol/mol lysine for lactoyl lysine, and 0.0135/0.041 mmol/mol lysine for glyceryllysine.

Statistical Evaluation—Data are expressed as mean ± S.D. The Student’s t test was used for statistical evaluation of significant differences between both groups.

RESULTS

Amide-AGEs in Human Blood Plasma of Uremic Patients versus Healthy Subjects—Plasma was obtained from 11 healthy subjects (controls) with normal renal function and no proteinuria and 18 HD patients. In dialysis patients, samples were obtained predialysis before the mid-week treatment session. Details on the study population are summarized in Table 3. Normal renal function was defined as a serum creatinine level below 102 μmol/liter. After appropriate dilution and separation from the protein residue by a 3 kDa molecular weight cutoff membrane, the plasma samples were subjected to liquid chromatography coupled with mass spectrometric detection using multiple reaction monitoring.

As shown in Fig. 1, plasma levels of all four carboxylic acid amides were significantly higher in HD patients than those in controls (acetyl lysine, 746 ± 151 versus 335 ± 63 pmol/ml plasma; formyl lysine, 281 ± 78 versus 145 ± 70 pmol/ml plasma; lactoyl lysine, 273 ± 90 versus 36 ± 15 pmol/ml plasma; glyceryllysine, 23.0 ± 5.1 versus 5.6 ± 0.9 pmol/ml plasma). To compare with well established AGE structures, we measured plasma concentrations of CML and CEL. In accordance with the literature (4), the levels were significantly elevated in

### Table 3

| Profile of subjects examined in this study | Healthy subjects | Hemodialysis patients |
|-------------------------------------------|------------------|-----------------------|
| No. of participants                        | 11               | 18                    |
| Age (years)                                | 66 ± 6           | 69 ± 11               |
| HbA1c (%)                                  | 5.6 ± 0.2        | 6.3 ± 1.7             |
| Serum creatinine (μmol/liter)              | 81 ± 8           | 706 ± 222 (k)         |
| C-reactive protein (mg/liter)              | 1.7 ± 1.1 (<1.0–4.5) | 23 ± 19 (6–70)       |

| N²-lactoyl lysine                          | 0.0007/0.002 mmol/mol lysine | 0.0013/0.004 mmol/mol lysine |
| formyl lysine                              | 0.001 mmol/mol lysine        | 0.0007/0.002 mmol/mol lysine |
| lactoyl lysine                             | 0.0135/0.041 mmol/mol lysine | 0.0007/0.002 mmol/mol lysine |

*Not significant.*

*p < 0.001 versus healthy subjects. [44352] JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 286 • NUMBER 52 • DECEMBER 30, 2011*
HD patients (CML, 2051 ± 760 versus 129 ± 59 pmol/ml plasma; CEL, 2063 ± 785 versus 157 ± 78 pmol/ml plasma).

In selected cases, the identity of the detected compounds was confirmed by comparison of the retention time and fragmentation pattern with authentic reference standards. The results for lactoyl lysine are shown in Fig. 2. The quasi molecular ion \([M + H]^+\) at \(m/z\) 219.2 is expected to undergo dehydration with immediate decarbonylation to the ion at \(m/z\) 173. The subsequent deamination of the immonium fragment renders the most abundant ion at \(m/z\) 156. Cyclization of lactoyl lysine to a six-membered ring and elimination of \(N^6\) functionality yields the ion at \(m/z\) 130. \(m/z\) 84 presents the pyrrolinium ion.

Formation of Amide-AGEs in α-Dicarbonyl/N\(^1\)-t-Boc-lysine Incubations—To identify the direct precursors of the carboxylic acid amides, 1-DG, 3-DG, β-arabinofuranos-2-ulose (glucosone) and methylglyoxal were incubated with \(N^6\)-t-Boc-lysine under physiological conditions (pH 7.4, 37 °C). To study the impact of oxygen, incubations were conducted under aera-
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TABLE 4
Comparison of amide concentrations in incubation mixtures of 200 mM glucose, 42 mM 1-desoxyglucoson (1-DG), 42 mM glucosone, and 42 mM methylglyoxal in the presence of 42 mM \( N^t \)-t-Boc-lysine and in incubation mixtures of 42 mM Amadori product under aerated and deaerated conditions

| Glucose\(^a\) | Amadori product\(^b\) | 1-DG\(^b\) | Glucose\(^a\) | Methylglyoxal\(^b\) |
|----------------|-----------------------|----------|----------------|-------------------|
|                | Aerated | Deaerated | Aerated | Deaerated | Aerated | Deaerated | Aerated | Deaerated |
| \( N^6 \)-acetyl lysine | 0.46    | 0.35    | 0.004   | 0.007     | 0.78    | 0.75    | 0.05    | 0.04     |
| \( N^6 \)-formyl lysine   | 1.39    | 0.48    | 0.37    | 0.05      | 1.47    | 1.08    | 2.60    | 0.89     |
| \( N^6 \)-lactoyl lysine  | 0.06    | 0.06    | < LOD   | < LOD     | 0.25    | 0.26    | < LOQ   | 0.35     |
| \( N^6 \)-glycerinyl lysine | 0.75    | 0.10    | 0.20    | < LOQ     | 0.15    | 0.09    | 0.06    | < LOQ    |

\(^a\) At 28 days.
\(^b\) At 72 h.

Formation of Amide-AGEs in Glucose/N\(^t\)-t-Boc-lysine Incubations—Because glucose is a major source of glycation and glycoxidation in vivo, incubations of glucose with \( N^t \)-t-Boc-lysine and of 1-desoxy-1-(\( N^6 \)-lysino)-D-fructose, the Amadori product of both, were performed under physiological conditions (pH 7.4, 37 °C) for comparison. The time course of formation of target amides in the glucose-lysine system over a time period of 4 weeks is shown in Fig. 3 and Table 4.

Acetyl lysine and formyl lysine were the dominant amide modifications under deaerated conditions. Both lactoyl lysine and glycerinyl lysine showed considerably lower levels of formation. In contrast to formyl and glycerinyl lysine, the impact of aeration on formation of acetyl and lactoyl lysine was negligible. However, the ratio of glycerinyl to formyl lysine changed dramatically with aeration: 1.50 versus 0.70 mmol/mol lysine compared with 0.16 versus 0.70 mmol/mol lysine with deaeration (incubation time, 48 days). This led to the conclusion that there must be additional oxygen-dependent mechanisms for glycerinyl lysine independent from formation of formyl lysine.

DISCUSSION

Performing several model incubations, we tried to gain insights into possible formation pathways linked to Maillard chemistry in vivo. \( N^6 \)-[2-[(5-amino-5-carboxypentyl)amino]-2-oxoethyl]lysine and \( N^6 \)-glycolyl lysine are formed via rearrangement reactions of glyoxal with lysine in contrast to the novel amide AGEs involving \( \beta \)-dicarbonyl fragmentations (Fig. 4). We already investigated the degradation of the \( \alpha \)-dicarboxyl 1-DG in the presence of \( N^t \)-t-Boc-lysine, identifying 1-DG as a direct precursor of the amide AGEs (7). Now, glucosone was found to be another potential precursor of formyl lysine, while degradation of 3-DG did not result in any acylation. This is expected because 3-DG in contrast to 1-DG and glucose cannot be converted to an \( \alpha \)-oxoenediyl, which is a prerequisite for \( \beta \)-dicarbonyl fragmentations. As shown in Fig. 5, all three \( C^6 \)-dicarbonyl structures are known Maillard degradation products of glucose. Although 1-DG and 3-DG are generated non-oxidatively via enolization and dehydration from the Amadori product, glucosone is formed via autoxidation of glucose or oxidation of the Amadori product or the intermediate Schiff base (31).

The results of incubations of glucose with \( N^t \)-t-Boc-lysine and of the Amadori product of both revealed a very similar formation pattern for acetyl lysine and lactoyl lysine in reaction mixtures with glucose and 1-DG, respectively (Table 4 and Fig. 3), confirming 1-DG as the direct precursor independent from oxidative processes via hydrolytic \( \beta \)-dicarbonyl fragmentation. Although the formation of formyl lysine from 1-DG responded to oxidative conditions, the difference was far more pronounced from glucose or the Amadori product. This pointed to the existence of another precursor emerging from oxidative pathways, which was identified as glucosone. Here, formyl lysine was detected in significant amounts, whereas formation of all other target amides was negligible. These findings were further supported by the same ratio of acetyl to formyl lysine in 1-DG versus glucose incubations under deaerated conditions, i.e., glucose contributes only under oxidative conditions to formation of formyl lysine from glucose, whereas 1-DG is the...
sole precursor in absence of oxygen. As formyl lysine, glycerinyl lysine showed a significant discrepancy in glucose versus 1-DG incubations induced by aeration. From the present results the additional reaction pathway leading to glycerinyl lysine from glucose remained unclear as none of the other Maillard intermediates responded. However, it is obvious that oxidation is required and that glucosone is not a relevant precursor.

Comparing the results of in vivo samples with above model experiments, incubations under aerated conditions should simulate the situation in uremia. Uremia has been described as a state of inflammatory stress resulting from either increased oxidation of carbohydrates and lipids (oxidative stress) or inadequate detoxification or inactivation of reactive carbonyl compounds derived from both carbohydrates and lipids by oxidative and non-oxidative chemistry (carbonyl stress) (3). Including only non-diabetic subjects in the present study, the impact of differences in glucose plasma concentrations was avoided.

The comparison gave a very diverse picture. Although all target amides in vivo were significantly increased in uremia (Fig. 1), only formyl and glycerinyl lysine responded to aeration in the glucose-lysine model. In addition, the ratio of concentrations between the single amides was completely different. In vivo, acetyl, formyl, and lactoyl lysine were within the same range, with glycerinyl lysine showing 10-fold lower concentrations. In contrast, glucose incubations showed the smallest concentrations for lactoyl lysine. Given that glycerinyl lysine indeed follows the patterns of the model incubations, this suggests additional pathways for the formation of acetyl, formyl, and lactoyl lysine. An alternative mechanism leading to lactoyl lysine in vivo is the reaction of methylglyoxal with ε-amino lysine residues corresponding to our identification of Nε-glycoloyl lysine in glyoxal lysine model incubations based on rearrangement reactions. Indeed, when we incubated methylglyoxal with Nε-t-Boc-lysine, we found significant amounts of lactoyl lysine independent from the presence of oxygen (Table 4). In vivo, this also explains the 7-fold increase in uremia, as we measured a 4-fold increase in methylglyoxal in such subjects (138 ± 39 versus 496 ± 132 pmol/ml plasma). Concentrations of acetyl lysine were highest in vivo and responded to renal failure by a 2-fold increase. This might be explained by increased cell death triggered by the strong inflammatory processes observed under uremia resulting in accelerated release of acetyl lysine from acetylated histone proteins. In addition, the increased concentration of methylglyoxal in presence of peroxynitrite might also contribute to the acetylation of ε-amino lysine residues in vivo. Massari et al. (32) described a mechanism of ε-lysine acetylation by a methylglyoxal-peroxynitrite system in vitro. Although the direct reaction of methylglyoxal with proteins probably dominates over that with peroxynitrite, the post-translational acetylation of proteins by radical mechanisms in the presence of methylglyoxal might be a plausible
second non-enzymatic pathway to acetyltransferase-catalyzed reactions. Moreover, although much less reactive, H$_2$O$_2$ could replace peroxynitrite in the acetyl-generating reaction from methylglyoxal. The idea to explain the 2-fold increase of formyl lysine in uremia based on the in vitro models is undermined by the fact that neither 1-DG nor glucosone have been detected in vivo so far. An intriguing alternative mechanism might be the with inflammation increased oxidative DNA breakdown to give 3'-formylphosphate-ended DNA fragments as a potential precursor, but again, such species have not been identified in vivo (23).

As a first attempt to assess the importance of the novel amide-AGEs as clinical markers for kidney failure, plasma levels of the well-established lysine modifications CML and CEL were measured additionally. In healthy human subjects, concentrations were within the same range. The AGE levels decreased in the following order: acetyl lysine ≈ CML > formyl lysine > lactoyl lysine > glycerinyl lysine. In uremia, we observed a 13- to 16-fold increase of CEL and CML. Amide-AGEs showed only a 2- to 8-fold increase. Thus, amide-AGEs might be of major importance when compared with other AGE structures identified in vivo so far, even though the impact of uremia on plasma concentrations of CML and CEL is more pronounced.

In conclusion, we previously proposed a formation mechanism for the novel amide-AGEs acetyl, formyl, lactoyl, and glycerinyl lysine via degradation of 1-DG in the presence of lysine (7). Now, glucosone and methylglyoxal were identified as alternative precursors for formyl and lactoyl lysine, respectively. All four amide-AGEs were unequivocally detected in human plasma, formyl, lactoyl, and glycerinyl lysine for the first time. The results strongly suggest a major participation of non-enzymatic Maillard mechanisms on amide-AGE formation pathways in vivo paralleled by enzymatic processes. The pathophysiologic consequences of two to 7-fold increased levels of amide-AGE free adducts in plasma of HD patients is not yet understood and requires further investigation. Also, the question which quantities of AGE-free adducts are derived from the breakdown of AGE-modified proteins or from direct synthesis remains unclear. However, absolute plasma concentration levels were in the same range similar to other established lysine modifications, i.e. CML and CEL, and suggest a comparable impact of non-enzymatic biochemistry on pathophysiology in the human organism.

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