Imidazolium crosslinks derived from reaction of lysine with glyoxal and methylglyoxal are increased in serum proteins of uremic patients: evidence for increased oxidative stress in uremia

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Abstract **Glyoxal (GO) and methylglyoxal (MGO) are reactive dicarbonyl compounds formed during autoxidation of both carbohydrates and lipids. They may react with lysine and arginine residues of proteins in Maillard or browning reactions, yielding advanced glycation or lipoxidation end products. Among these are the imidazolium crosslinks, *N*,*N*(-di(*N*$^{\varepsilon}$-lysino))imidazolium (glyoxal-lysine dimer, GOLD) and *N*,*N*(-di(*N*$^{\varepsilon}$-lysino))-4-methyl-imidazolium (methylglyoxal-lysine dimer, MOLD). We have detected and measured GOLD and MOLD in human serum by electrospray ionization/mass spectrometry/mass spectrometry (ESI/MS/MS), using $^{15}N_4$-GOLD and $^{15}N_4$-MOLD as internal standards. In this report we show that levels of GOLD and MOLD are significantly elevated (3–4-fold, $P < 0.01$) in sera of non-diabetic uremic patients, compared to age-matched controls, and represent a major class of non-enzymatic, Maillard reaction crosslinks in plasma proteins. These results provide strong evidence for increased non-enzymatic crosslinking of tissue proteins by GO and MGO in uremia, implicating oxidative stress and resultant advanced glycation and lipoxidation reactions in tissue damage in uremia.**

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Key words: Advanced glycation end product; Crosslink; Glyoxal; Imidazole; Maillard reaction; Methylglyoxal

1. Introduction

Advanced glycation and lipoxidation end products (AGEs, ALEs) are formed non-enzymatically during Maillard or browning reactions of proteins with reducing sugars or lipid peroxidation products [1,2]. AGEs and ALEs accumulate in long-lived tissue proteins, such as collagens and lens proteins, and are thought to have a role in the development of pathology in aging [3], diabetes [4,5], vascular disease [6] and chronic renal failure [7]. In previous work, we have shown by both ELISA and chemical assays that protein-bound AGEs or ALEs, including *N*$^{\varepsilon}$-(carboxymethyl)lysine (CML) [8,9], pyrraline [10], pentosidine [11] and malondialdehyde-lysine (unpublished) are increased in serum proteins of uremic and hemodialysis patients, suggesting a relationship between advanced glycation and lipoxidation reactions and the pathophysiology of renal disease.

Reactive dicarbonyl compounds, such as glyoxal (GO) and methylglyoxal (MGO), are critical intermediates in the chemical modification of proteins during autoxidation of reducing sugars and polyunsaturated fatty acids [1,2,12]. Thornalley has reported increased levels of the MGO in blood in diabetes and has proposed that MGO is not only an important mediator of tissue damage in diabetes, atherosclerosis, oxidative stress and aging, but that MGO-modified proteins are also recognized by macrophage receptors involved in removal of modified proteins from the body [13]. Although the majority of products remain uncharacterized, reaction of GO and MGO with proteins leads to formation of imidazolone adducts [13,14] and *N*$^{\varepsilon}$-(carboxyalkyl)lysines, such as CML [15–17] and the homologous compound *N*$^{\varepsilon}$-(carboxyethyl)lysine (CEL) [18]. In model systems, Wells-Knecht and colleagues also identified the imidazolium crosslinks known as glyoxal-lysine dimer (GOLD) and methylglyoxal-lysine dimer (MOLD) [19,20] (insets to Fig. 1). More recently, Nagaraj et al. [21] reported the identification of MOLD in human serum and detected increased levels of MOLD in serum of diabetic patients using an HPLC method.

In this report we describe a more specific and selective electrospray ionization/mass spectrometry/mass spectrometry (ESI/MS/MS) procedure for simultaneous measurement of both GOLD and MOLD in serum proteins, and report that both of these compounds are present in human serum proteins, that they are present at similar concentrations, that they are significantly increased in serum of uremic and hemodialysis patients, and that GOLD and MOLD represent the major non-enzymatic crosslinks in serum proteins in uremia. We propose that increased chemical modification of serum and tissue proteins by dicarbonyl compounds may be important in the pathogenesis of vascular disease in uremia.

2. Materials and methods

2.1. Preparation of standards

$^{15}N_4$-GOLD and $^{15}N_4$-MOLD were synthesized from $^{15}N_2$-formyllysine ($^{15}N_2$-fL), prepared by reaction of $^{15}N_2$-lysine with acetic anhydride and formic acid, as described by Hofmann et al. [22]. To prepare $^{15}N_4$-GOLD, $^{15}N_2$-fL (9 mg, 50 mmol) was added to 30 ml of 3.4 M GO solution (100 mmol) and 10 ml 37% formaldehyde (100 mmol). The mixture was heated at 65°C, with additional GO and formaldehyde (50 mmol each) added 8 times at hourly intervals. MOLD was prepared similarly, except that the reaction was conducted at 37°C. After 30 h, products were deformylated by mixing with one-half volume of concentrated HCl (final concentration: 4 M HCl) and heating

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Abbreviations: AGE, advanced glycation end product; ALE, advanced lipoxidation end product; GO, glyoxal; MGO, methylglyoxal; GOLD, glyoxal-derived di-lysine imidazolium crosslink; MOLD, methylglyoxal-derived di-lysine imidazolium crosslink; ESI, electrospray ionization; MS, mass spectrometry; SIM, selected ion monitoring; CEL, *N*$^{\varepsilon}$-(carboxyethyl)lysine; CML, *N*$^{\varepsilon}$-(carboxymethyl)lysine; fL, formyllysine

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for 2 h at 100°C. Amino acid analysis of an aliquot of the hydrolysate indicated 40–50% conversion of $^{15}N_2$-fL to GOLD or MOLD. The resulting brown solutions were dried by centrifugal evaporation and redissolved in distilled water. The majority of the brown contaminants were removed by applying the aqueous solution to a Supelco C-18 cartridge (3 ml) and eluting with 30% acetonitrile in water. To isolate basic compounds, the eluate was evaporated and dissolved in distilled water (5 ml), then applied to a 1 ml column of SP-Sephadex (Pharmacia), equilibrated with distilled water. The column was washed with 20 ml of 0.05 M HCl, then GOLD or MOLD eluted with 5 ml of 1 M HCl. After drying by centrifugal evaporation and dissolving in amino analysis buffer, the GOLD and MOLD were applied to a cation exchange amino acid analysis system and fractions containing the cross-links were collected, desalted on Dowex-50-H^+, and dried. The purities of GOLD and MOLD ($>85\%$) were checked by amino acid analyzer. The only detectable contaminants were ammonia and free lysine.

2.2. Patients

Serum samples were obtained from 30 subjects: 15 chronic renal failure (CRF) patients (six males and nine females with non-diabetic nephropathy; mean age, 58 ± 4 years); and 15 normal controls (eight males, seven females; mean age, 55 ± 5 years). The serum levels of creatinine in uremic patients and healthy subjects were 11.3 ± 0.5 mg/dl (mean ± S.D., $n = 15$) and 0.5 ± 0.3 mg/dl ($n = 15$), respectively.

2.3. Sample preparation

To quantify protein-bound GOLD and MOLD in human serum, the enzyme digested samples were prepared as previously described [10,23]. Briefly, to 1 ml plasma from each patient, 1 ml cold 10% trichloroacetic acid was added and mixed. Precipitated proteins were pelleted by centrifugation, and then 2 ml diethyl ether was added to the pellet and mixed. Ether layer was separated by centrifugation and the resultant pellet was dried for lyophilization. To 10 mg of lyophilized protein from each sample, 0.02 mg peptidase (Sigma, from porcine intestine, 2%, w/w) was added in 1 ml PBS and incubated for 16 h at 37°C in a shaker incubator. Secondary digestion was continued for additional 8 h with the addition of different peptidase (Sigma, from *Streptomyces griseus*, 2%, w/w). The last digestion was achieved by the addition of pronase E (Sigma, 2%, w/w, 16 h). After digestion, samples were filtered through a 0.22 μm filter (Millipore, USA) for ESI/MS/MS analysis. Recovery of $^{15}N_4$-GOLD and $^{15}N_4$-MOLD spiked

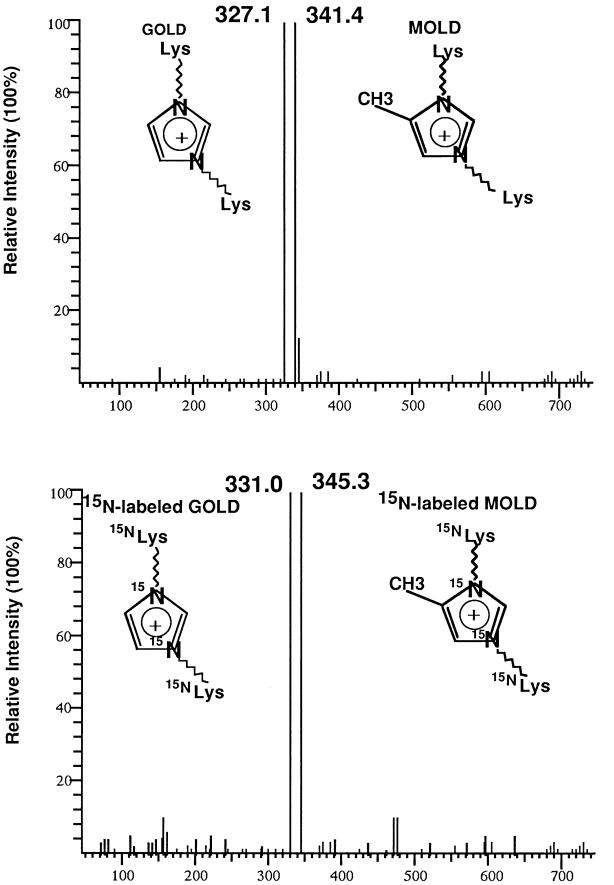

Fig. 1. ESI mass spectra of GOLD (glyoxallysine dimer), MOLD (methylglyoxallysine dimer), $^{15}N_4$-GOLD and $^{15}N_4$-MOLD.

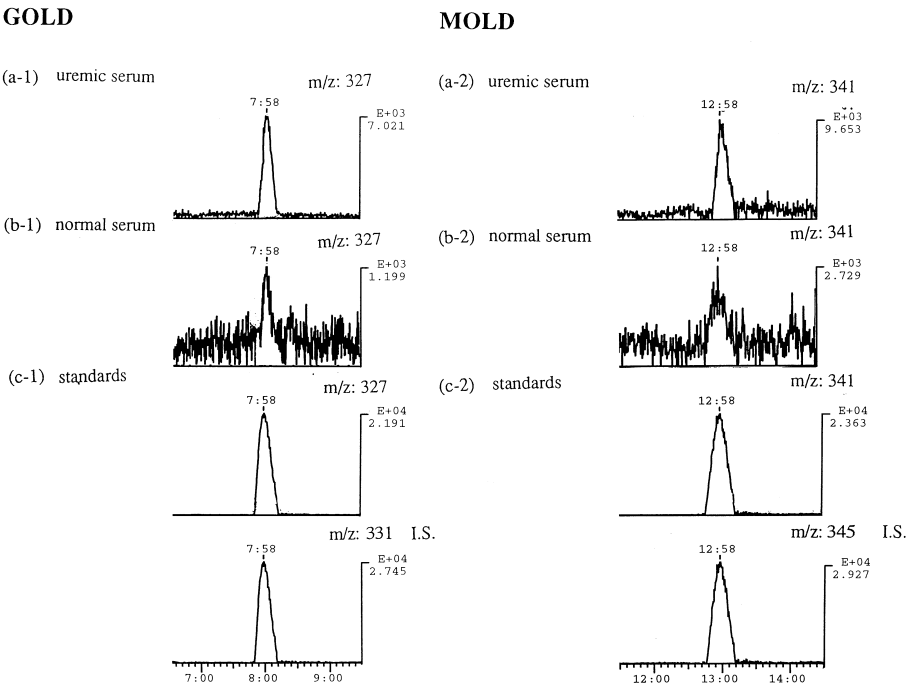

Fig. 2. ESI/SIM chromatograms of uremic sera (a-1, -2); normal sera (b-1, -2); standards (c-1, -2). (a-1), (b-1) and (c-1) show GOLD, and (a-2), (b-2) and (c-2) show MOLD. $^{15}N_4$-GOLD and $^{15}N_4$-MOLD, used as internal standards, show peaks at *m/z* 331 and *m/z* 345, respectively.

into protein samples at a concentration of 500 ng/ml was 97.5 ± 4.8% (mean ± S.E.M., $n = 4$).

2.4. Mass spectrometry

GOLD and MOLD were resolved by reversed-phase high performance liquid chromatography (RP-HPLC) and analyzed by ESI/MS/MS using a TSQ 7000 triple quadrupole mass spectrometer (Finnigan MAT Instruments, USA). RP-HPLC was conducted on a Monitor C-18M column (5 mm, 150×4.6 mm ID, Column Engineering, USA) equilibrated with solvent A (0.03% TFA in H_2O), and eluted with a linear gradient to 50% solvent B (40% acetonitrile, 60% H_2O, 0.02% TFA) during the first 30 min; the column was then washed with 50% B for 20 min, with 100% B for 5 min, then re-equilibrated in 100% solvent A for 20 min; flow rate was 0.3 ml/min. Elution times for GOLD and MOLD were approximately 8 and 13 min. For MS/MS analysis, the ionizing energy, spray current and voltage were 72 eV, 1.5 mA, and 4.5 kV, respectively. For quantification of GOLD and MOLD, the protonated molecular ion intensities *m/z* 327/331 (GOLD/$^{15}N_4$-GOLD) and *m/z* 341/345 (MOLD/$^{15}N_4$-MOLD) were compared in the selected ion monitoring mode. GOLD and MOLD concentrations were normalized as pmol/mg serum protein, measured by the Lowry assay [24]. For confirmation of structure, daughter ions (positive ions) were monitored by selected reaction monitoring (SRM) using a collision gas pressure of 2 mT helium and collision energy of −25 eV.

2.5. Statistical analysis

Data were analyzed by the unpaired Student's *t*-test and the results are expressed as means ± S.D.

3. Results

Fig. 1 shows ESI full scan mass spectra of GOLD, MOLD, $^{15}N_4$-GOLD and $^{15}N_4$- MOLD. The spectra show the intact protonated molecular ions, $[M+H]^+$ for GOLD (*m/z* 327), MOLD (*m/z* 341), $^{15}N_4$-GOLD (*m/z* 331), and $^{15}N_4$-MOLD (*m/z* 345).

No significant fragment or adduct ions were found in the ESI spectrum in the mass range from 100 to 700. Based on measurement of their intense parent molecular ions (*m/z* 327 and 341), both GOLD and MOLD were detected in normal

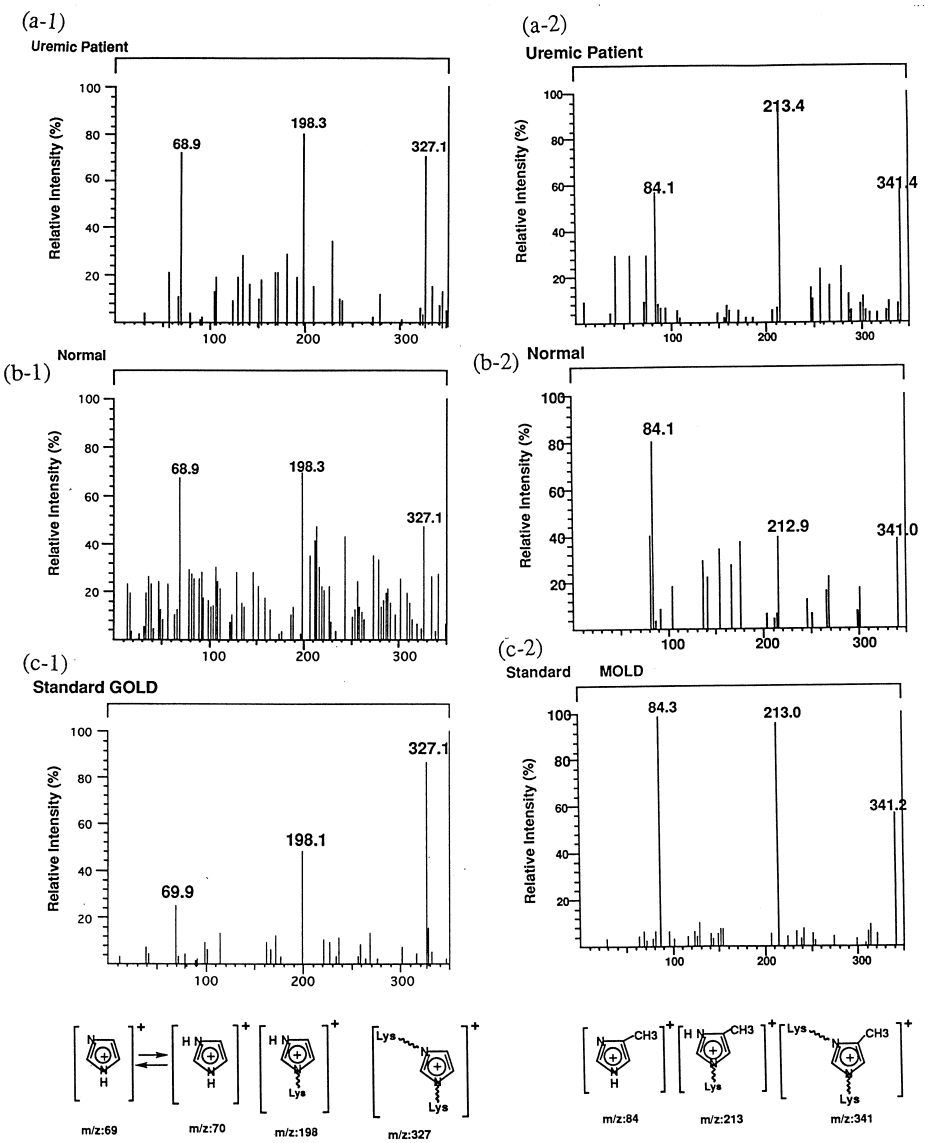

Fig. 3. ESI/MS/MS spectra of GOLD and MOLD in uremic sera (a-1, -2), normal sera (b-1, -2) and standard GOLD and MOLD (c-1, -2). The structures proposed for the daughter ions are shown under the figures.

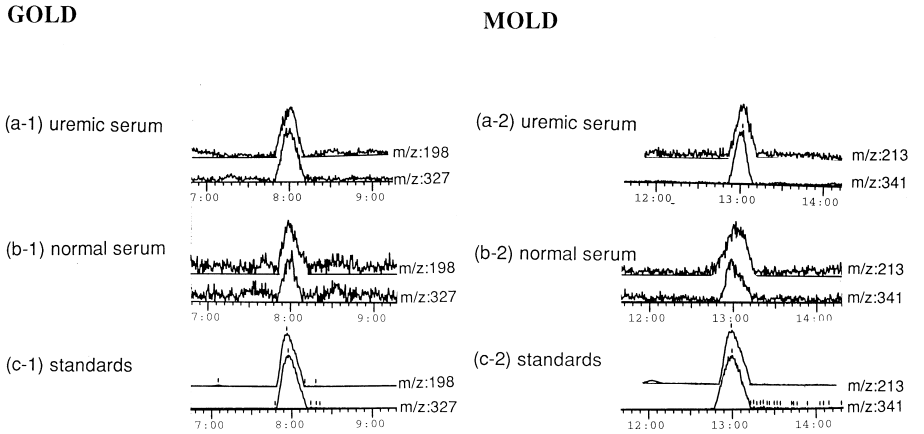

Fig. 4. ESI/SRM chromatograms of MOLD and GOLD in uremic sera (a-1, -2), normal sera (b-1, -2) and of natural GOLD and MOLD standards. The *m/z* 327 and *m/z* 341 are the protonated molecular parent ion of GOLD and MOLD, respectively, and the *m/z* 198 and *m/z* 213 correspond to their major protonated daughter ions. Proposed structures are as shown in Fig. 3.

and uremic serum by ESI/SIM, and quantified by comparison to the intensity (peak area) of *m/z* 331 and *m/z* 345 ions of the parent ions in the internal standards.

Fig. 2 shows representative ESI/SIM chromatograms of uremic sera (a-1, -2), normal serum (b-1, -2), and standards (c-1, -2), illustrating the detection of GOLD and MOLD in human serum proteins, and increased levels of these compounds in serum proteins from uremic patients. Collision gas induced dissociation of the parent ions of GOLD and MOLD, *m/z* 327 and 341, respectively, yielded daughter ion spectra with a base peak at *m/z* 69 and major fragment at *m/z* 198 for GOLD, and a base peak at *m/z* 84 and fragment ion of *m/z* 213 for MOLD (Fig. 3). Despite some differences in relative ion intensities resulting from differential ion suppression, daughter ion spectra characteristic of GOLD and MOLD standards were readily apparent in products detected in uremic and normal serum.

As shown in Fig. 4, when the daughter ions were measured by selected reaction monitoring, the traces of the daughter ions in GOLD and MOLD in serum samples closely followed those of the daughter ions produced by GOLD and MOLD standards, further supporting the specificity of the ESI/MS/MS assay. Quantitative analyses by ESI/SIM (Fig. 5) showed that GOLD and MOLD were present at similar levels in serum proteins of control, non-uremic patients, and that they were comparably increased by 4–5-fold in uremic patients.

4. Discussion

Both GO and MGO react with proteins by a variety of pathways, leading to *N*-(carboxyalkyl)amino acids, imidazolones and imidazolium salts [15,16,18–20].

Among these compounds, we have identified the crosslinks, GOLD and MOLD, in serum proteins using a highly specific and sensitive assay, and shown that these compounds are significantly increased in serum proteins from uremic patients. Our estimates of MOLD are significantly lower than those of Nagaraj et al. [21], however minor contaminants in protein hydrolysates can contribute to the apparently higher concentrations of MOLD detected by HPLC alone. In contrast, the LC/MS/MS method, which combines HPLC with selected ion monitoring MS/MS, provides much greater specificity for detection and quantitation of MOLD. Comparisons with previous studies indicate that GOLD and MOLD are present at much higher concentrations in serum proteins than the fluorescent crosslink pentosidine. Thus, levels of pentosidine in serum proteins range from about 5 pmol/mg in normal to 25 pmol/mg in uremic serum [11], less than 10% the combined concentrations of GOLD and MOLD in the same sources. At this point, the imidazolium crosslinks constitute the primary Maillard reaction crosslinks which have been measured in plasma proteins.

As with pentosidine, the origin of GOLD and MOLD in serum protein in uremia is unknown. Since glucose concentration was not increased in the serum of patients studied in this project, the increase in GOLD and MOLD cannot be attributed to glucose. There were no significant difference in LDL concentration between the control and uremic groups. Although there was a significant increase in serum triglycerides in uremic patients [25,26], there was no significant correlation ($P > 0.05$) between these measurements and levels of GOLD or MOLD. Overall, these results suggest a general increase in oxidative stress in uremia, independent of changes in blood glucose or lipids. It is possible that GOLD and MOLD may be derived directly from GO and MGO in plasma, but concentrations of these precursors in uremic plasma are also unknown. Alternatively, they may be derived from reactions of ascorbate or dehydroascorbate with proteins, since both CML and CEL are formed from ascorbate under

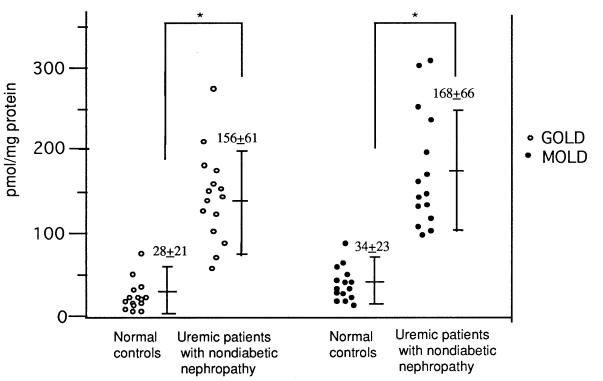

Fig. 5. Serum levels of GOLD and MOLD in uremic patients with non-diabetic nephropathy and in normal subjects. Data represent means ± S.D. for 15 patients. $^*P < 0.005$.

oxidative conditions [18]. In this case, an increase in the ratio of dehydroascorbate:ascorbate in plasma in uremia, resulting from increased oxidative stress, might predispose to the formation of GOLD and MOLD. Finally, the increase in GOLD and MOLD might occur at constant glycemia and triglyceridemia, by increased autoxidation of both carbohydrate and lipid substrates as a result of a general increase in oxidative stress or decrease in glutathione-dependent antioxidant defenses in uremia. Measurement of blood concentrations of GO and MGO, ratios of dehydroascorbate:ascorbate, and evaluation of the effects of supplementation with anti-oxidants and glutathione precursors or enhancers (*N*-acetylcysteine or lipoic acid) may lead to an understanding of the origin of increased GOLD and MOLD in uremia.

At this time, because of their low concentrations in serum proteins, there is no reason to believe that GOLD and MOLD in these proteins are of any direct pathological significance.

However, they are biomarkers of altered biochemistry in uremia and an understanding of their biological and chemical origin may lead to better appreciation of the underlying mechanisms of pathogenesis and to development of improved therapeutic strategies for the management of uremic patients.

The use of ESI/MS/MS for measurement of these and other Maillard products in plasma proteins will be important for accurate, sensitive and specific measurement of these and other chemical modifications of plasma proteins during aging and in disease.

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