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Increase in Three α,β-Dicarbonyl Compound Levels in Human Uremic Plasma: Specific *in Vivo* Determination of Intermediates in Advanced Maillard Reaction

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Methylglyoxal (MGO), glypxal (GO) and 3-deoxyglucosone (3-DG) are reactive α,β-dicarbonyl intermediates in advanced Maillard reaction, which form advanced glycation and oxidation end products (AGEs) by reaction with both lysine and arginine residues in protein. We measured these three dicarbonyl compound levels in human plasma to estimate the relationship between accumulation of α,β-dicarbonyl compounds and AGE formation reactions in uremia and diabetes in human plasma by a highly selective and specific assay, electrospray ionization liquid chromatography mass spectrometry (ESI/LC/MS). We show that 3-DG and MGO levels are significantly higher in uremia and diabetes compared with age-matched healthy controls. Only the GO level in uremic plasma is significantly higher compared to diabetes and healthy controls. In both diabetic and uremic patients, these dicarbonyl compounds promote AGE accumulation *in vivo,* and especially in uremic patients, increased accumulation of GO could result from accelerating oxidative stress. © 1999 Academic Press

Nonenzymatic glycation, lipoxidation and the advanced Maillard reaction have been proposed to play a role in the pathogenesis of end-stage renal disease (1) as well as diabetic complications (2, 3) and the aging process (4), by forming various protein modifications, such as DNA adducts and crosslinking among amino acids (5). In the process of above these reactions, some dicarbonyl compounds are found *in vitro* and *in vivo.* 3-deoxyglucosone (3-DG) is produced from the multiple dehydration and rearrangement of Amadori compounds in the early stage Maillard reaction (6, 7, 8), methylglyoxal (MGO) is produced by nonenzymatic fragmentation of triose phosphates (9), and glyoxal (GO) is formed by autoxidation of reducing sugars and polyunsaturated fatty acids (10, 11). These three a,b-dicarbonyls form advanced glycation and oxydation end products (AGEs) *in vitro,* and have been postulated to be a major source of intracellular and plasma AGEs (9). We previously reported the accumulation of some AGEs in human uremic plasma (12, 13). In this study, we have detected and measured these three dicarbonyl compounds in human plasma at the same time by a highly selective and specific assay, electrospray ionization liquid chromato-graphy mass spectrometry (ESI/LC/MS), and we show that among these three dicarbonyls the level of GO is especially elevated in non-diabetic uremic human plasma. This result indicates that the reactive oxygen generation system works on patients with non-diabetic chronic renal failure as a more important pathogenetic factor than advanced glycation reactions.

MATERIALS AND METHODS

Patients. Plasma samples were obtained from 55 living subjects: 20 patients with chronic renal failure (CRF) patients (mean age, 59 ± 7 years), 20 patients with non-insulin dependent diabetes mellitus (mean age, 61 ± 7 yers), and 15 normal subjects (mean age, 56 ± 7 years). CRF was attributed to chronic glomerulonephritis and non-diabetes. The serum levels of creatinine in uremic patients, in diabetic patients and healthy subjects were 12.1 ± 0.5 mg/dl (mean ± SD, $n = 20$), 1.2 ± 0.5 mg/dl (mean ± SD, $n = 20$) and 0.7 ± 0.3 mg/dl ($n = 15$), respectively. The serum samples were kept at −20°C prior to analysis.

Formation of 3-DG, MGO, and GO derivatives through reaction with 2,3-diaminonaphthalene (2,3-DAN). 3-DG was kindly supplied by Dr. K. Nakamura (Nippon Zoki Co, Ltd), MGO was purchased from Sigma and further purified by distillation under reduced pressure (14), and GO was purchased from Nacalai Tesque Inc. Formation of these dicarbonyl compounds' derivatives through reaction with 2,3-diaminonaphthalene (2,3-DAN) was performed according to the method of S. Miyata *et al.* (15). Briefly, 1 ml of 3-DG, MGO and GO solution in 10 mM phosphate buffer (pH 7.4) was incubated with 100 μl of 0.1% 2,3-DAN (Wako Pure Chemicals, Japan) at 4°C in the presence of 50 μl of 0.01% 3,4-hexanedione (Tokyo Kasei Organic Chemicals, Japan) as internal standard. The reaction was performed

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overnight at 4°C. The reaction mixture was extracted by 4 ml of ethyl acetate, and the solvent was dried under nitrogen atmosphere. The dried extract was reconstituted with 200 μl of methanol and filtered through a 0.22 μm filter (Millipore, USA) for ESI/LC/MS analysis. Recovery of 3,4-hexanedione spiked into plasma samples at a concentration of 500 ng/ml was 94.5 ± 6.5% (mean ± SE, $n = 4$).

Mass spectrometry. All the derivatized dicarbonyl compounds were resolved by reversed-phase high performance liquid chromatography (RP-HPLC) and analyzed by ESI/MS using a TSQ7000 triple stage quadrupole mass spectrometer (Thermoquest, USA). RP-HPLC was conducted on a Excelpak SIL-C18 column (5 μm, 150 × 4.6 mm ID, Yokogawa, Japan) equilibrated with solvent (40% methanol, 60% H2O, 0.2% acetic acid), and eluted with isocratic system at a flow rate of 0.4 ml/min. For MS/MS analysis, the ionizing energy, spray current and voltage were 72 eV, 1.5 mA, and 4.5kV, respectively. Quantitation of 3-DG, MGO and GO were performed by calculating a peak area ratio of each dicarbonyl-derived protonated molecular ion peak intensity (3-DG; *m/z* 285, MGO; *m/z* 195 and GO; *m/z* 181) to a protonated molecular internal standard ion peak intensity (3,4-hexanedione; *m/z* 223) in the selected ion monitoring mode (SIM). To confirm their structures, daughter ions (positive ions) were trapped and monitored by electrospray/mass spectrometry/mass spectrometry (ESI/MS/MS) system using a collision gas pressure of 2 mT Helium and collision energy of −25 eV.

Quantitation analysis of the three dicarbonyl componds was done according to each protonated molecular ion peak area ratio obtained by ESI/SIM spectra. The correlation coefficient between the added 3-DG standard concentration and the peak area (*m/z:* 285) ratio was 0.994 (Regression equation: $y = 2.5847x - 0.003027$, with standard concentration range from 1 nmol/ml to 600 nmol/ml); MGO standard concentration and the peak area (*m/z:* 195) ratio was 0.993 (Regression equation: $y = 0.98562x - 0.003931$, with standard concentration range from 1 nmol/ml to 600 nmol/ml); GO standard and the peak area (*m/z:* 181) ratio was 0.998 (Regression equation: $y = 1.2609x - 0.00228$, with standard concentration range from 1 nmol/ml to 600 nmol/ml).

RESULTS

Identification of 3-DG, MGO and GO derivatives with 2,3-DAN in human plasma. To identify and determine the levels of several dicarbonyl compounds in human serum on the same time analysis, we used mass spectrometry (ESI/LC/MS) with high selectivity and accuracy, employing the derivatization method by 2,3-DAN (Fig. 1) (15). These reactions were done quantitatively, and each derivative was stabl in ESI/MS analysis. Figure 2 shows ESI/SIM chromatograms of 2,3-DAN-adducts with 3-DG (*m/z:* 285)(a-1,-2), MGO (*m/z:* 195)(b-1,-2), GO (*m/z:* 181)(c-1,-2), and 3,4-hexanediones as internal standard (*m/z:*237, d-1,-2), based on their protonated molecular ions. It is shown that chromatograms of the extract from normal human plasma represented in a-2, b-2, c-2 and d-2 correspond to the chromatograms from their standard samples represented in a-1, b-1, c-1 and d-1 in Fig. 2. Collision gas-induced dissociation of the parent ions of each compound, *m/z:* 285, *m/z:* 195, and *m/z:* 181, respectively, yielded daughter ion spectra (Fig. 3). Although there are some differences in relative ion intensities resulting from differential ion suppression, daughter ion mass spectra of human plasma sample a-2, b-2, and c-2 almost match those of standard samples represented in a-1, b-1, and c-1 shown in Fig. 3. We confirmed the existence of these dicarbonyl compounds from the above data.

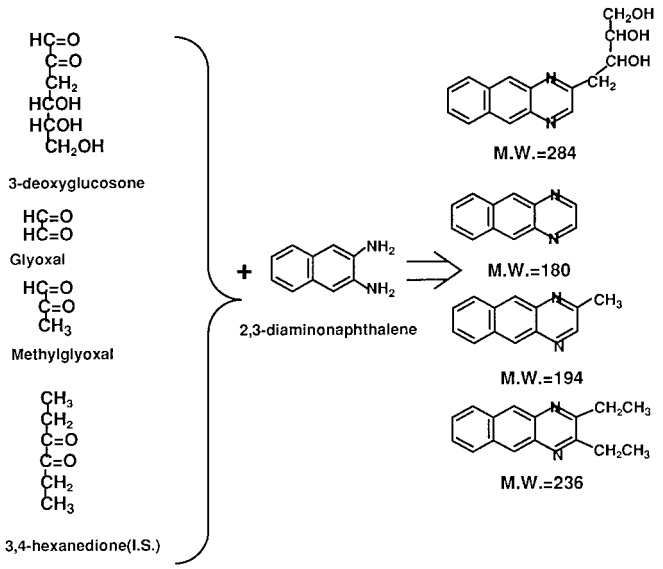

FIG. 1. Derivatization of α,β-dicarbonyl compounds using 2,3-diaminonaphthalene for ESI/MS measurment.

Plasma 3-DG, MGO and GO level in normal subject, diabetic, and uremic patients. As shown in Table 1, the plasma levels of 3-DG in diabetic mellitus (DM) patients and uremic patients were significantly elevated over normal controls ($p < 0.001$). The plasma levels of MGO were also significantly higher in DM and uremic patients than in normal controls ($p < 0.001$). When comparing the levels in DM and uremic patients, plasma of DM patients showed a significant accumulation of both 3-DG and MGO levels in plasma; but in plasma levels of GO, the accumulation in uremic patients was much higher than in both DM patients and normal controls ($p < 0.001$). However, there was no difference in the plasma GO level between DM patients and normal controls.

DISCUSSION

3-DG, MGO, and GO react with proteins by various pathways, leading to N-(carboxyalkyl) amino acids, imidazolones and imidazolium salts (16–18). 3-DG is well mentioned as an important intermediate in AGE formation from Amadori rearrangement product of Maillard glycation reaction, and is rapidly reduced by 3-DG reductases to form 3-deoxy-fructose, which is normally excreted to urine (19). Moreover MGO formed by nonenzymatic fragmentation of triose phosphate is metabolized to the unreactive D-lactate by the glyoxalase I and II (9, 20). Both 3-DG and MGO are widely acknowledged to be related to diabetic complications (14, 15, 21). GO is formed in the first step of autoxida-

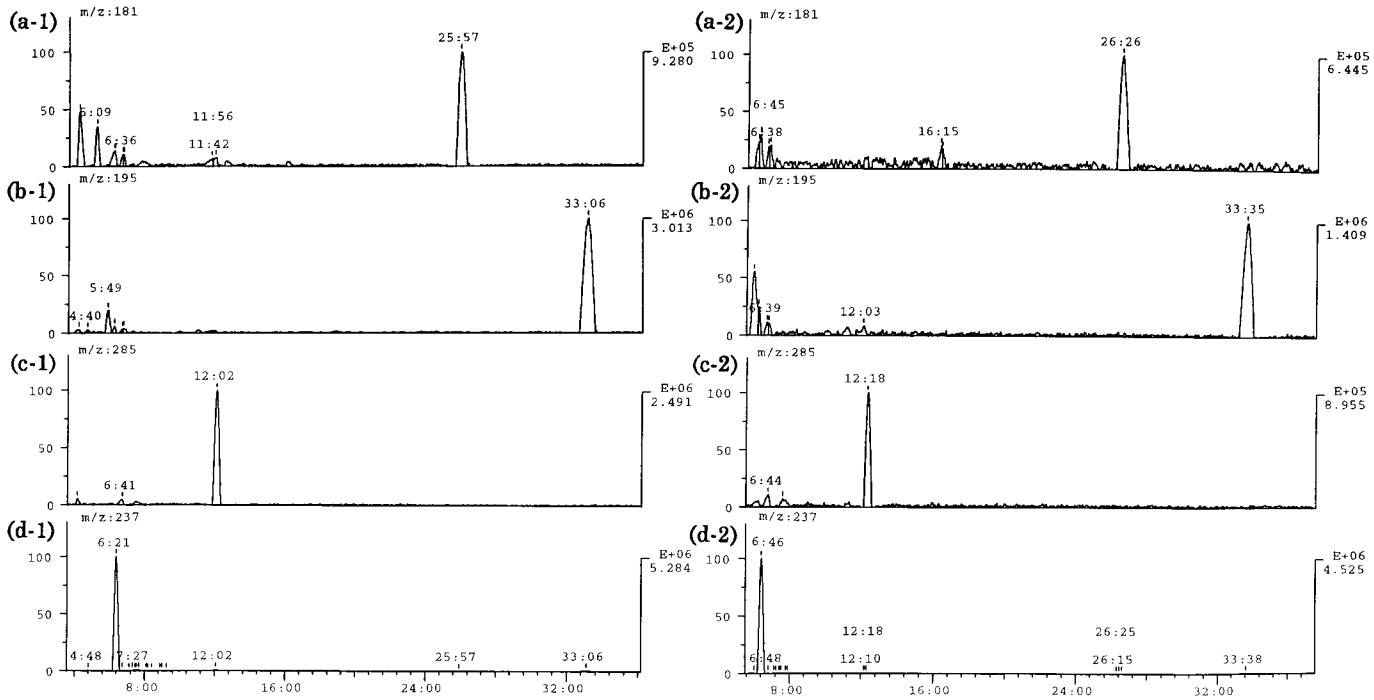

FIG. 2. ESI/SIM chromatograms of authentic 2,3-diaminonaphthalene (2,3-DAN) adducts derived from standard GO (a-1), MGO (b-1), 3-DG (c-1) and 3,4-hexanedione (d-1) as internal standard added to a normal control human plasma (each 100 ng/ml). ESI/SIM chromatograms of 2,3-DAN adducts derived from an uremic patient plasma are shown *m/z* 181 (a-2); *m/z* 195 (b-2); *m/z* 285 (c-2); *m/z* 237 (d-2):*m/z* 181 shows the protonated GO-2,3-DAN adduct, *m/z* 195 shows the protonated MGO-2,3-DAN adduct, *m/z* 285 shows the protonated 3,4-hexanedione-2,3-DAN adduct.

tion glycosylation and crosslinking of the proteins (16, 17). It is also reported that GO is produced by lipid per-oxidation systems (22) and induces mutations by glyoxal-DNA adducts (23). GO and 3-DG are also reported to accelerate formation of AGE product in rat sensory neurons (24). Thus, these α,β-dicarbonyl compounds may be involved in steps toward many age-related human diseases.

In this study, we first determined serum 3-DG, MGO and GO levels at the same time by ESI/LC/MS with high selectivity and accuracy. Our results indicate that 3-DG and MGO levels in diabetic plasma are significantly higher than in uremic plasma or normal human plasma, but there is not a difference in the mean plasma level of GO between diabetic patients and normal controls. Plasma levels of 3-DG and MGO in uremic patients increased significantly over normal controls, and GO accumulation was far higher than in diabetic and normal plasmas. The mean serum levels of 3-DG in CRF patients measured by gas chromatography/mass spectrometry (GC/MS) reported by Niwa et al were much higher than in diabetic patients (21). Our estimated levels of 3-DG in uremic patients measured by ESI/LC/MS were less than in diabetic patients. This inconsistency may be caused from the difference of analytical method of 3-DG between GC and LC. These α,β-dicarbonyl compounds are considered to be unstable in heated condition at atmosphere and exist very small amount in human serum. The increased accumulation of 3-DG in diabetes may be caused by hyperglycemia (25). The uremic patients with diabetes showed significantly higher serum concentrations of 3-DG than those without diabetes, and in uremic patients without diabetes the content of 3-DG may be related with the lack of the enzyme activity of 3-DG reductases in the end-stage kidney (26). Recent study has demonstrated a highly oxidative stress and the availability of precursors such as oxidized form of ascorbic acid enhanced AGE formation in uremic patients (27).

Our results in this study suggest that in uremic patients, the accumulation of AGEs occurs to undergo the advanced glycoxidation reaction rather than just

TABLE 1

3-DG, MGO and GO Levels in Human Plasma

	3-DG (ng/ml)	MGO (ng/ml)	GO (ng/ml)
DM patients (n = 20)	81 ± 20	158 ± 46	78 ± 28
Uremic patients (n = 20)	59 ± 13	110 ± 18	221 ± 28
Normal controls (n = 15)	26 ± 16	47 ± 12	67 ± 20

Note. Data are means ± S.D.

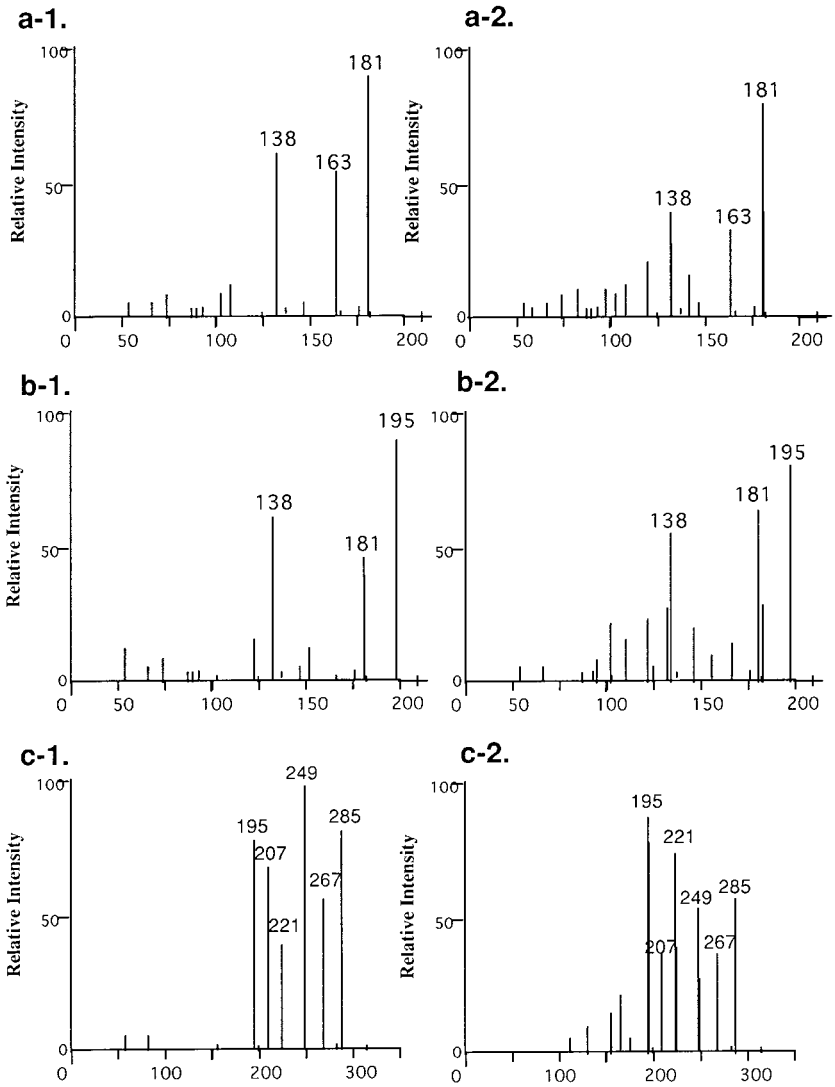

FIG. 3. ESI/MS/MS spectra of 2,3-DAN adducts derived from standard GO (a-1), MGO (b-1) and 3-DG (c-1) added to a normal control human plasma (each 100 ng/ml), and normal human plasma (a-2), (b-2) and (c-2).

that of the advanced glycation reactions. Thus, the plasma protein in uremia might be much more exposed to oxidative stress than in diabetes. It is reported that the production of superoxide in uremic patients was more accelerated than in normal controls (28). As the autoxidation of glucose might be more accelerated in uremic plasma than in diabetic plasma, the increased plasma level of GO is only seen in uremic patients. Recently, we identified and quantitated one of the AGEs, imidazolium salts, glyoxallysine dimer (13), and demonstrated its increased level in uremic plasma. It will be derived from increased accumultion of glyoxal *in vivo*. The biological and physiological significances of GO and glyoxal lysine dimer in uremia are unclear, but these dicarbonyl compounds will be important biomarkers of biochemistry in uremia and diabetic complications.

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