Generation of Superoxide Anions during the Reaction of Guanidino Compounds with Methylglyoxal

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Uremic toxins are accumulated in the blood of patients with chronic renal failure (CRF), although alteration of the toxicity by the interaction of various uremic retention products has not been precisely clarified. In this study, we found that cytochrome c added to incubation mixtures containing guanidino compounds and methylglyoxal in phosphate buffer solution (pH 7.4) resulted in reduction of cytochrome c. Superoxide anions were generated from incubation mixtures of each guanidino compound with methylglyoxal, because the reduction was inhibited by the addition of superoxide dismutase. The incubation mixture containing each guanidino compound and methylglyoxal had different rates of generation of the superoxide anion from other mixtures. A relatively higher superoxide anion formation rate was observed in the incubation mixture containing Arg and methylglyoxal ($7.9 \pm 0.5\,\mathrm{nmol \cdot m^{-1} \cdot min^{-1}}$), or in the incubation mixture containing methylguanidine and methylglyoxal ($6.3 \pm 0.6\,\mathrm{nmol \cdot ml^{-1} \cdot min^{-1}}$). These findings suggest that interactions of various uremic retention products which accumulate in the blood of uremic patients may generate reactive oxygen species and may be involved in the oxidative stress observed in CRF patients. The addition of aminoguanidine, which is known to inhibit the formation of advanced glycation end products, to a mixture of guanidino compounds and methylglyoxal inhibited reactions between guanidino compounds and methylglyoxal.

Key words guanidino compound; superoxide; methylglyoxal; diketone; chronic renal failure; aminoguanidine

The Maillard reaction is a nonenzymatic reaction of glucose with albumin which forms a relatively stable Amadori product. The Amadori product undergoes a series of further reactions over a period of several months to years, and the reactions form reactive intermediates that finally lead to stable advanced glycation end products (AGE).[1—3] Methylglyoxal is an endogenous metabolite.[4] Moreover, it was identified as a precursor of AGE[5—7] which accumulates in the plasma of uremic patients at a reported concentration of $1.53 \pm 0.25\ \mu$M.[8] This compound is known to react with the guanidino group in proteins[4,7,9,10] and it binds and reversibly modifies arginine residues.[11—13] The reactions have been shown to occur even at physiological concentrations of methylglyoxal.[14] Natural guanidino compounds, substances that have a guanidino group, have been identified in animal tissues.[15] Hyperargininemic patients have 10- to 15-fold higher L-arginine (Arg) levels in their serum.[16] Moreover, many guanidino compounds accumulate in the blood of patients with chronic renal failure (CRF). Typical guanidino compounds are shown in Fig. 1. The concentrations of guanidino compounds in the serum of CRF patients were reported to be: Arg, $111 \pm 19.7\ \mu$M; methylguanidine (MG), $6.68 \pm 3.98\ \mu$M; *N*-acetyl-L-arginine (*N*-Ac-Arg), 1.52 ± 0.66; beta-guanidinopropionic acid (GPA), 0.025—$0.45\ \mu$M; guanidine (G), $2.93 \pm 1.42\ \mu$M; creatinine (CTN), $868 \pm 218\ \mu$M; guanidinoacetic acid (GAA), $1.96 \pm 0.64\ \mu$M; creatine (CT), $51.8 \pm 44.6\ \mu$M; and guanidinosuccinic acid (GSA), $37.4 \pm 19.6\ \mu$M (mean±S.D., $n=13$).[17] Guanidino compounds are uremic toxins and therefore candidates for markers of renal dysfunction since they are more toxic than CTN or blood urea nitro-

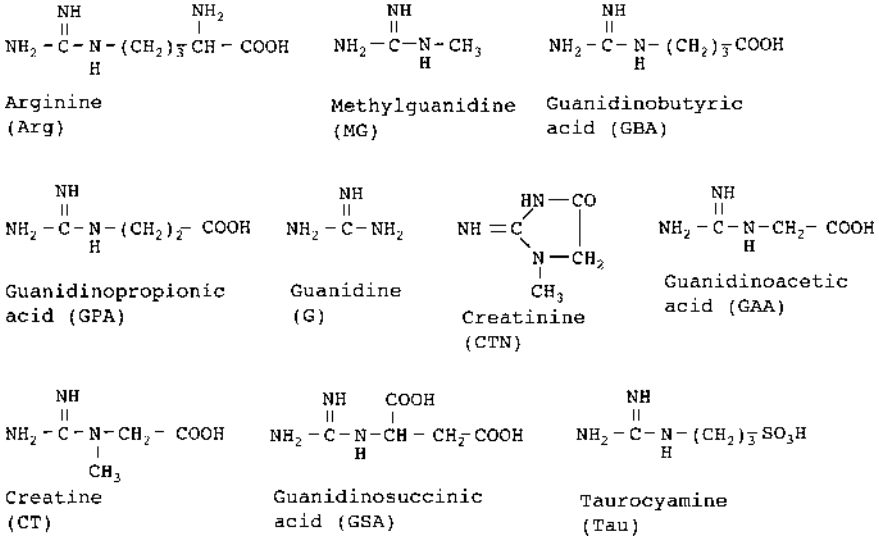

Fig. 1. Structure of Guanidino Compounds

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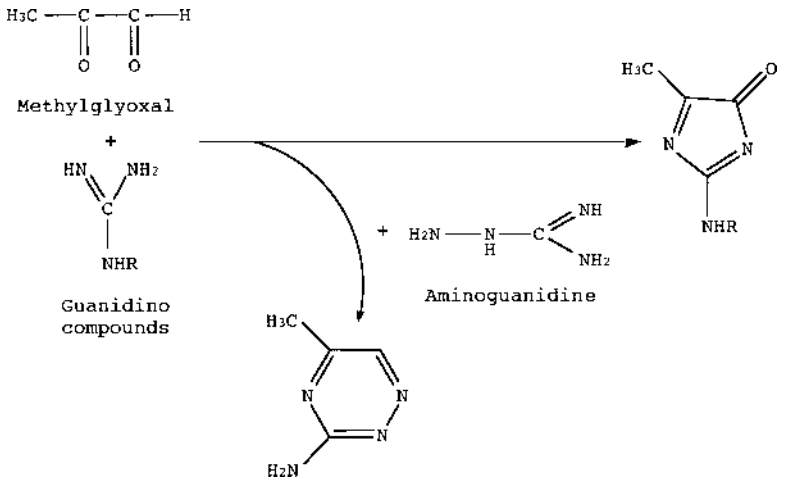

Chart 1

gen (BUN).[18,19] However, the toxicity is low and synergistic because serum concentrations of guanidino compounds are far lower than those of CTN or BUN.

CRF patients exhibit oxidative stress,[20] and reactive oxygen species have been implicated in the progressive loss of renal function.[21] However, the relationship between reactive oxygen species and the interaction of various uremic retention products has not been precisely clarified. Ortwerth *et al.* demonstrated cytochrome c reduction in incubation mixtures of a variety of carbohydrates and *N*-acetyl-lysine or *N*-Ac-Arg in the presence of a chelator.[22] Interactions of some compounds called uremic toxins, which accumulated in the blood of uremic patients,[23—25] may generate more toxicity than reactive oxygen species.

In the present study, we determined the generation of superoxide anions from reaction mixtures of guanidino compounds with methylglyoxal and in addition the effect of the reaction between guanidino compounds and methylglyoxal by aminoguanidine (AG) (Chart 1). These findings indicate a mechanism for generating reactive oxygen species under physiological conditions.

Experimental

Materials Water was purified using a MILLI-Q Labo from Nihon Millipore Kogyo (Yonezawa, Japan). CT and 1-methylguanidine hydrochloride were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Disodium hydrogenphosphate dodecahydrate, sodium diphosphate decahydrate, diethylenetriaminepentaacetic acid (DTPA), Arg, guanidine hydrochloride, *N*-Ac-Arg dihydrate, taurocyamine (TAU), cytochrome c, and AG bicarbonate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Gamma guanidinobutyric acid (GBA), GAA, GPA, GSA, and CTN were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Superoxide dismutase (SOD) from *Bacillus stearothermophilus* was purchased from Seikagaku (Tokyo, Japan). Methylglyoxal was purchased from Nacalai Tesque (Kyoto, Japan).

Analytical Methods Mixtures containing 10 mM of various guanidino compounds, 10 mM methylglyoxal, and DTPA 50 μM with or without 10 mM AG in 50 mM phosphate buffer (pH 7.4) were incubated for 48 h at 37 °C. One milliliter of incubation mixture was mixed with 2 ml of cytochrome c solution containing 3.0 mg/ml cytochrome c and DTPA 50 μM in 50 mM phosphate buffer (pH 7.4). Superoxide formed was calculated as the difference in the increase in absorbance at 550 nm with or without 200 units of SOD at 18 °C. The molar extinction coefficients (ε_{mM}=29.9) given by Massey[26] were used.

Electron Spin Resonance Conditions Reactive oxygen species were analyzed as spin adducts of 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). Five microliters of 8.9 M DMPO were added to 100 μl of incubation mixture and 100 μl of 0.5 M sodium hydroxide, dispersed with a vortex mixer, and immediately transferred to a quartz flat cell for electron spin resonance (ESR) analysis. ESR spectra were measured using a JES-REIX spectrometer (JEOL, Tokyo, Japan). The conditions were as follows: microwave frequency, 9.42 GHz; microwave power, 8.0 mW; magnetic field, 332.6 mT; modulation width, 0.06 mT at 300.0 kHz modulation frequency; response time, 0.3 s; and sweep time, 5.0 mT/2 min at 25 °C.

Spectrophotometric Study The fluorescence intensities of the diluted incubation mixtures were measured. Incubation mixture 150 μl was added to 3 ml of purified water. UV absorbances were measured using a spectrophotometer, for which the incubation mixtures were also diluted. Incubation mixture 100 μl was added to 3 ml of purified water.

Determination of Guanidino Compounds The triketone ring of ninhydrin was previously demonstrated to be cleaved by alkali to give *o*-carboxyphenylglyoxal with a dicarbonyl structure,[27] which reacts with guanidines resulting in the production of fluorescent derivatives.[28] Guanidino compounds were assayed using the modified HPLC method according to the method of Nohara *et al.*[19] The eluent was a mixture of 5 mM sodium dihydrogenphosphate solution (pH 4.6) containing 5 mM 1-octanesulfonic acid sodium salt, 5 mM ninhydrin, and 2-propanol (85 : 15 v/v%) at a flow rate of 1.0 ml/min. One microliter of incubation mixture was injected, and guanidino compounds were separated on the column (Senshu Pak PEGASIL ODS, 150×4.6 mm i.d., particle size 5 μm). The eluate from the separation column was mixed with 500 mM sodium hydroxide solution at a flow rate of 0.5 ml/min. The mixture was reacted in a coil (5 m×0.5 mm i.d., 95 °C), and the fluorescence intensity was monitored with the detector (excitation at 395 nm, emission 500 nm).

Results

Generation of Superoxide Anions from the Reaction of MG and Methylglyoxal The reduction of cytochrome c

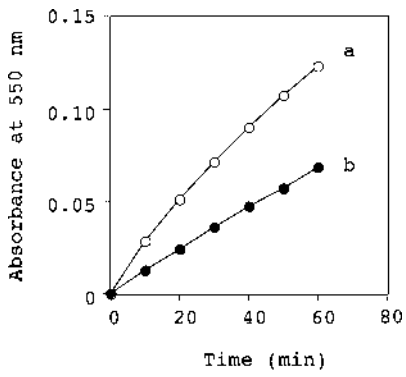

Fig. 2. Effect of Addition of SOD to Incubation Mixtures of MG and Methylglyoxal on the Serial Changes in UV Absorbance of Cytochrome c

(a) Without SOD; (b) with 200 units of SOD.

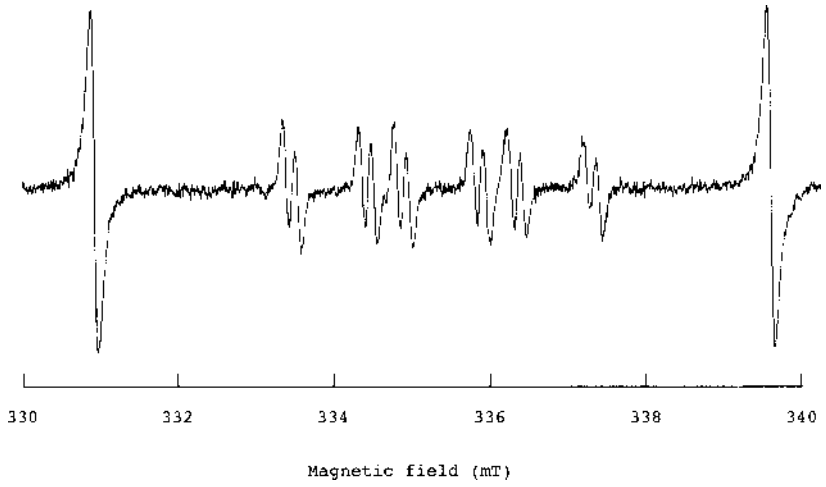

Fig. 3. ESR Spectra of the DMPO-Spin Adduct of Superoxide Anions from an Incubation Mixture of MG and Methylglyoxal under Alkaline Conditions (pH 12.3)

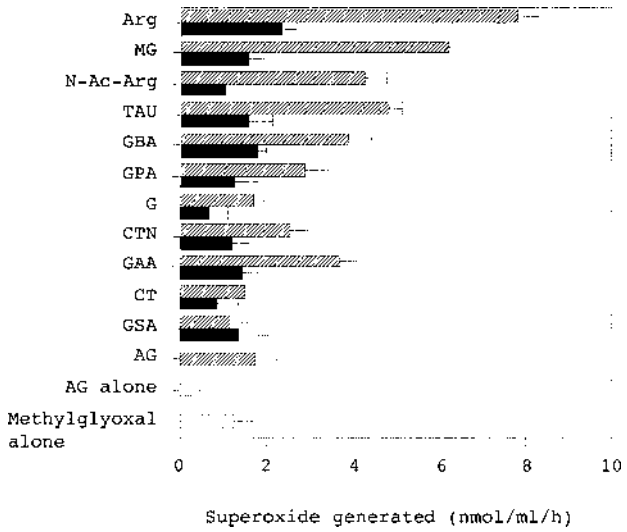

Fig. 4. Generation of Superoxide Anions from Incubation Mixtures of Various Guanidino Compounds and Methylglyoxal with or without AG

Each value represents the mean±S.D. (n=4). ■, incubation mixtures with AG; ▨, without AG; □, incubated single solutions.

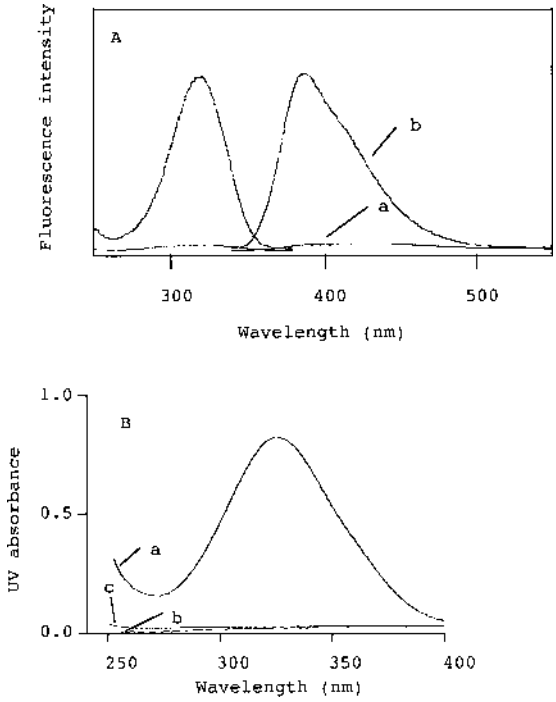

Fig. 5. (A) Excitation and Emission Spectra of Fluorescence from Incubation Mixtures of MG and Methylglyoxal with (a) or without AG (b)
(B) UV Spectra from Incubation Mixtures of MG and Methylglyoxal with (a) or without AG (b), and from AG Solution Alone (c)

was observed with or without SOD over 60 min. Figure 2 shows the serial changes in UV absorbance when SOD was added the incubation mixture of MG and methylglyoxal at 37 °C for 2 d. SOD suppressed the cytochrome c reduction rate to 44% at 60 min.

The signal of the DMPO-spin adduct of superoxide anions was observed, as shown in Fig. 3.

Effect of Incubation with AG on the Generation of Superoxide Anions Mixtures containing guanidino compounds and methylglyoxal incubated for 2 d at 37 °C showed superoxide anion generation rates different from other mixtures (Fig. 4). A relatively higher formation rate of superoxide anion was observed in the incubation mixture containing Arg and methylglyoxal, or in that containing MG and methylglyoxal. In addition, a relatively lower formation rate of superoxide anion was observed in the incubation mixture containing CT and methylglyoxal, or in the incubation mixture containing GSA and methylglyoxal. In contrast, incubation mixtures with AG showed similar generation rates.

Spectrophotometric Study of the Formation of Derivatives by the Reaction of Various Guanidino Compounds or AG with Methylglyoxal Fluorescent materials were found in incubation mixtures without AG but not in mixtures incubated with AG. Figure 5A shows excitation and emission spectra of fluorescence from incubation mixtures of MG and methylglyoxal with or without AG.

There were differences in fluorescence intensities and UV absorbance in incubation mixtures with or without AG. Figure 5B shows UV spectra from incubation mixtures of MG and methylglyoxal with or without AG, and from a solution

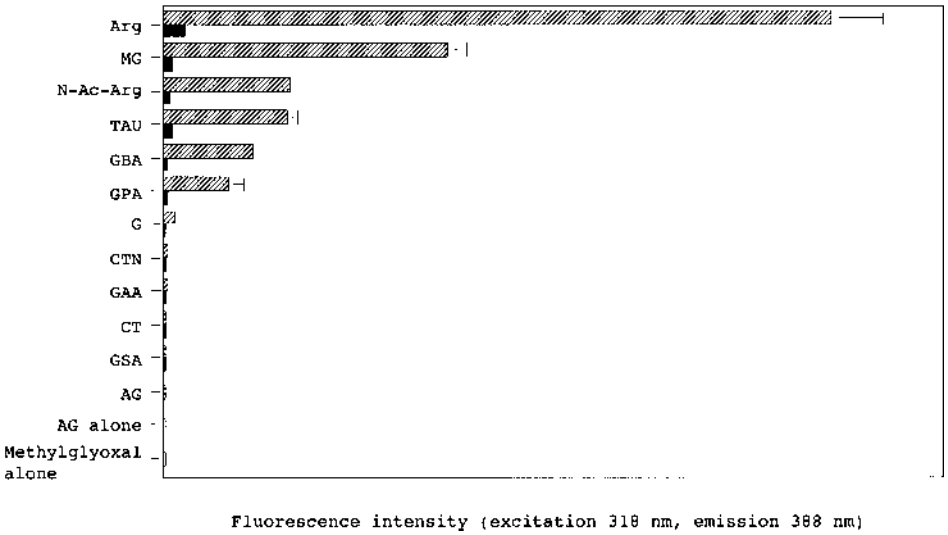

Fig. 6. Fluorescence Intensities from Reaction Mixtures of Guanidino Compounds and Methylglyoxal with or without AG

The fluorescence intensity at excitation 318 nm and emission 388 nm was measured. Each value represents the mean±S.D. (n=4). ■, incubation mixtures with AG; ▨, without AG; □, incubated single solutions.

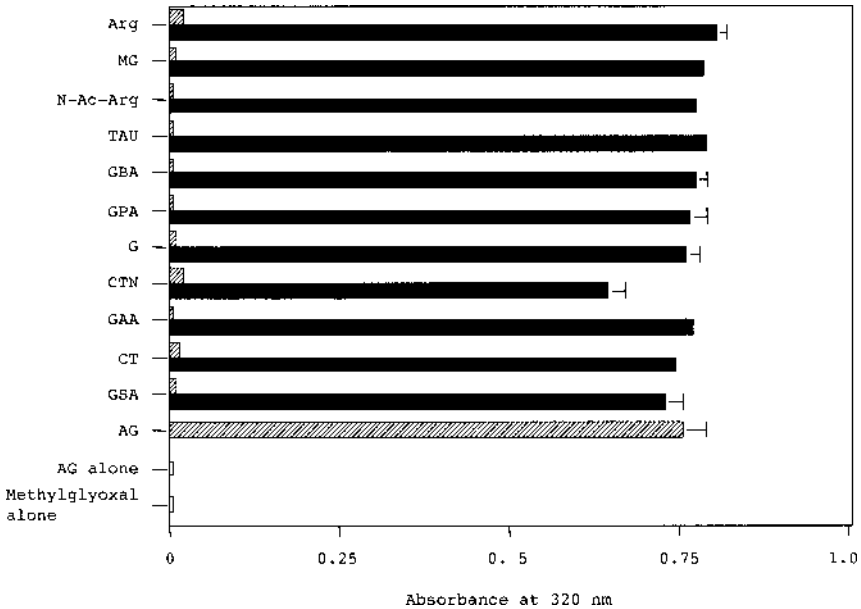

Fig. 7. UV Absorbance from Reaction Mixtures of Guanidino Compounds and Methylglyoxal with or without AG

The UV absorbance at 320 nm was measured. Each value represents the mean±S.D. (n=4). ■, incubation mixtures with AG; ▨, without AG; □, incubated single solutions.

containing AG alone. There was no increase in UV absorbance at 320 nm in the incubation mixture without AG and in the solution with AG alone. However, there was an increase in the mixture incubated with AG, which was based on characteristics of the formation of 1,2,4-triazine products, not based on UV absorbance by AG itself.

Fluorescence intensities with excitation at 318 nm and emission at 388 nm were measured in reaction mixtures of various guanidino compounds and methylglyoxal with or without AG (Fig. 6). The fluorescence of the incubation mixture containing Arg and methylglyoxal showed high intensity, and that of the incubation mixture containing MG and methylglyoxal was similarly high. However, high fluorescence intensity was not found in all mixtures incubated with AG. Incubation mixtures without AG not only had high generation rate of superoxide anions as shown in Fig. 4, but also showed comparatively high fluorescence intensity with excitation at 318 nm and emission at 388 nm. Guanidino compound slightly remained in the incubation mixtures with high fluorescence intensities. The percentage of the guanidino compounds remaining in the incubation mixtures with methylglyoxal were: Arg, 11.8; MG, 19.1; *N*-Ac-Arg, 24.4; TAU, 28.1; GBA, 25.4; GPA, 23.5; G, 33.7; CTN, 24.0; GAA, 24.1; CT, 5.3; and GSA, 31.8 (%; n=2).

UV absorbances at 320 nm were measured in reaction mixtures of various guanidino compounds and methylglyoxal with or without AG (Fig. 7). UV absorbance occurred at 320 nm in all incubation mixtures of various guanidino compounds and methylglyoxal with AG, and the absorbance values were similar. However, no absorbance was seen in any

mixture incubated without AG. Moreover, guanidino compounds largely remained in the incubation mixtures of various guanidino compounds and methylglyoxal with AG (%, $n=2$; Arg, 61.6; MG, 63.2; *N*-Ac-Arg, 79.0; TAU, 76.2; GBA, 76.0; GPA, 76.3; G, 73.9; CTN, 49.9; GAA, 75.3; CT, 61.6; GSA, 81).

Discussion

Ferricytochrome c is reducible to ferrocytochrome c by superoxide.[29] However, it is thought that the reduction of cytochrome c during the glycation reaction may occur by superoxide and by direct reduction from the Amadori compound.[22] Therefore superoxide anion generation during the glycation reaction has been confirmed by the SOD-dependent reduction of cytochrome c,[30—33] and the amount of superoxide formed was calculated.[22] In the present study, the incubation mixture of MG and methylglyoxal in the presence of DTPA as a chelator was found to reduce cytochrome c, and the reduction was effective in the presence of SOD. The superoxide assay was done in the absence of transition metal ions, because transition metal may accelerate superoxide anion generation from the Amadori compound.[34] Moreover, ESR spectra showed the DMPO-spin adduct of superoxide anions. Therefore superoxide anions were found to be generated from incubation mixtures with MG and methylglyoxal in phosphate buffer under physiological conditions (pH 7.4, 37 °C), and the rate of generation was higher than the total generation by the incubation mixture of MG or methylglyoxal alone. Similarly, generation of superoxide anions occurred in incubation mixtures of various guanidino compounds and methylglyoxal. These incubation mixtures contained fluorescent materials because methylglyoxal reacted with the guanidino group of guanidino compounds to form imidazolone adducts.[4,35] The incubation mixtures with high fluorescence intensities showed comparatively high generation rates of superoxide anions. Therefore it is considered that each fluorescent material generated superoxide anions.

AG is known to an inhibitor of AGE formation.[36—39] AG reacts with biological compounds with carbonyl groups,[40] and therefore it was suggested that AGE formation is inhibited by reaction with Amadori-derived compounds, including 3-deoxyglucosone.[40] In the present study, it was found that methylglyoxal reacted earlier with AG than guanidino compounds. Generation rates of superoxide anions from incubation mixtures with AG showed similar values. Moreover, methylglyoxal reacts with the guanidino group of guanidino compounds to form imidazolone adducts with fluorescence at excitation 318 nm and emission 388 nm, while it reacts with the amino groups of AG to form 3-amino-1,2,4-triazine derivatives with UV absorbance at 320 nm.[41] UV absorbance was found at 320 nm in the incubation mixtures of various guanidino compounds and methylglyoxal with AG, while no fluorescence was found at excitation 318 nm, emission 388 nm. In addition, in excess of 60% of guanidino compounds generally remained in the incubation mixtures, except for CTN which was converted to CT. These findings indicate that AG had higher reactivity with methylglyoxal than guanidino compounds.

The generation of superoxide anions from the reaction of guanidino compounds or AG with methylglyoxal may occur *in vivo*, and may be involved in the oxidative stress observed in CRF patients. However, it is considered that superoxide anions generated from the reaction may be not harmful because of the lower generation rate compared with that occurring endogenously in cells.[42] It is important to clarify the mutual interactions of various uremic retention products that accumulate in the blood of uremic patients, because the reaction of guanidino compounds with methylglyoxal generates more toxic superoxide anions than guanidino compounds alone.

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References

1) Ikeda K., Higashi T., Sano H., Jinnouchi Y., Yoshida M., Araki T., Ueda S., Horiuchi S., *Biochemistry*, **35**, 8075—8083 (1996).
2) Makita Z., Bucala R., Rayfield E. J., Friedman E. A., Kaufman A. M., Korbet S. M., Barth R. H., Winston J. A., Fuh H., Manogue K. R., *Lancet*, **343**, 1519—1522 (1994).
3) Gugliucci A., Bendayan M., *Diabetologia*, **39**, 149—160 (1996).
4) Uchida K., Khor O. T., Oya T., Osawa T., Yasuda Y., Miyata T., *FEBS Lett*., **410**, 313—318 (1997).
5) Wells-Knecht K. J., Zyzak D. V., Litchfield J. E., Thorpe S. R., Baynes J. W., *Biochemistry*, **34**, 3702—3709 (1995).
6) Fu M. X., Requena J. R., Jenkins A. J., Lyons T. J., Baynes J. W., Thorpe S. R., *J. Biol. Chem*., **271**, 9982—9986 (1996).
7) Nukaya H., Inaoka Y., Ishida H., Tsuji K., Suwa Y., Wakabayashi K., Kosuge T., *Chem. Pharm. Bull*., **41**, 649—653 (1993).
8) Odani H., Shinzato T., Matsumoto Y., Usami J., Maeda K., *Biochem. Biophys. Res. Commun*., **256**, 89—93 (1999).
9) Shapiro R., Cohen B. I., Shiuey S. J., Maurer H., *Biochemistry*, **8**, 238—245 (1969).
10) Nagaraj R. H., Shipanova I. N., Faust F. M., *J. Biol. Chem*., **271**, 19338—19345 (1996).
11) Takahashi K., *J. Biol. Chem*., **243**, 6171—6179 (1968).
12) Takahashi K., *J. Biochem.* (Tokyo), **81**, 395—402 (1977).
13) Takahashi K., *J. Biochem.* (Tokyo), **81**, 403—414 (1977).
14) Lo T. W., Westwood M. E., McLellan A. C., Selwood T., Thornalley P. J., *J. Biol. Chem*., **269**, 32299—32305 (1994).
15) Robin Y., Marescau B., "Guanidines," ed. by Mori A., Cohen B. D., Lowenthal A., Plenum Press, New York, 1985, pp. 383—438.
16) Terheggen H. G., Lowenthal A., Colombo J. P., "Urea Cycle Diseases," ed. by Lowenthal A., Mori A., Marescau B., Plenum Press, New York, 1982, pp. 111—119.
17) De Deyn P. P., Robitaille P., Vanasse M., Qureshi I. A., Marescau B., *Nephron*, **69**, 411—417 (1995).
18) Nagase S., Aoyagi K., Sakamoto M., Narita M., Tojo S., *Nephrol. Dial. Transplant*., **3**, 790—794 (1988).
19) Nohara Y., Hanai T., Suzuki J., Matsumoto G., Iinuma F., Kubo H., Kinoshita T., Watanabe M., *Biol. Pharm. Bull*., **23**, 1015—1020 (2000).
20) Tepel M., Echelmeyer M., Orie N. N., Zidek W., *Kidney Int*., **58**, 867—872 (2000).
21) Yamada H., Yamada Y., Adachi T., Fukatsu A., Sakuma M., Futenma A., Kakumu S., *Nephron*, **84**, 218—223 (2000).
22) Ortwerth B. J., James H., Simpson G., Linetsky M., *Biochem. Biophys. Res. Commun*., **245**, 161—165 (1998).
23) Bergstrom P., Furst P., Abstracts of Papers, Uremic toxins. Proc. 7th Int. Congr. Nephrol., Montreal, Canada, 1978, pp. 669—675.
24) Minkoff L., Gaertner G., Darab M., Mercier C., Levin M. L., *J. Lab. Clin. Med*., **80**, 71—78 (1972).
25) Horowitz H. I., Stein I. M., Cohen B. D., White J. G., *Am. J. Med*., **49**, 336—345 (1970).
26) Massey V., *Biochim. Biophys. Acta*, **34**, 255—256 (1959).
27) Ruhemann J., *J. Chem. Soc*., **97**, 2025—2031 (1910).
28) Rex B. C., Richard B. D., *Nature* (London), **183**, 1053—1055 (1959).
29) Butler J., Jayson G. G., Swallow A. J., *Biochim. Biophys. Acta*, **408**, 215—222 (1975).
30) Sakurai T, Tsuchiya S., *FEBS Lett*., **236**, 406—410 (1988).
31) Gillery P., Monboisse J. C., Maquart F. X., Borel J. P., *Diab. Metabol*., **14**, 25—30 (1988).

32) Mullarkey C. J., Edelstein D., Brownlee M., *Biochem. Biophys. Res. Commun*., **173**, 932—939 (1990).
33) Yim H. S., Kang S. O., Hah Y. C., Chock P. B., Yim M. B., *J. Biol. Chem*., **270**, 28228—28233 (1995).
34) Mossine V. V., Linetsky M., Glinsky G. V., Ortwerth B. J., *Chem. Res. Toxicol*., **12**, 230—236 (1999).
35) Thornalley P. J., *Gen. Pharmacol. Rev*., **27**, 565—573 (1996).
36) Makita Z., Vlassara H., Rayfield E., Cartwright K., Friedman E., Rodby R., Cerami A., Bucala R., *Science*, **258**, 651—653 (1992).
37) Kihara M., Schmelzer J. D., Poduslo J. F., Curran G. L., Nickander K. K., Low P. A., *Proc. Natl. Acad. Sci. U.S.A*., **88**, 6107—6111 (1991).
38) Hammes H. P., Brownlee M., Edelstein D., Saleck M., Martin S., Federlin K., *Diabetologia*, **37**, 32—35 (1994).
39) Soulis-Liparota T., Cooper M., Papazoglou D., Clarke B., Jerums G., *Diabetes*, **40**, 1328—1334 (1991).
40) Edelstein D., Brownlee M., *Diabetes*, **41**, 26—29 (1992).
41) Thornalley P. J., Yurek-George A., Argirov O. K., *Biochem. Pharmacol*., **60**, 55—65 (2000).
42) Chance B., Sies H., Boveris A., *Physiol. Rev*., **59**, 527—605 (1979).