Free Radical Research, 2003 Vol. 37 (2), pp. 205–211

Identification of the Binding Site of Methylglyoxal on Glutathione Peroxidase: Methylglyoxal Inhibits Glutathione Peroxidase Activity via Binding to Glutathione Binding Sites Arg 184 and 185

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Accepted by Professor J. Yodoi

(Received 23 August 2002; In revised form 5 September 2002)

Methylglyoxal (MG), a physiological α-dicarbonyl compound is derived from glycolytic intermediates and produced during the Maillard reaction. The Maillard reaction, a non-enzymatic reaction of ketones and aldehydes with amino group of proteins, contributes to the aging of proteins and to complications associated with diabetes. In our previous studies (Che, *et al.* (1997) "Selective induction of heparin-binding epidermal growth factor-like growth factor by MG and 3-deoxyglucosone in rat aortic smooth muscle cells. The involvement of reactive oxygen species formation and a possible implication for atherogenesis in diabetes". *J. Biol. Chem.*, **272**, 18453–18459), we reported that MG elevates intracellular peroxide levels, but the mechanisms for this remain unclear. Here, we report that MG inactivates bovine glutathione peroxidase (GPx), a major antioxidant enzyme, in a dose- and time-dependent manner. The use of BIAM labeling, it was showed that the selenocysteine residue in the active site was intact when GPx was incubated with MG. MALDI-TOF-MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) and protein sequencing examined the possibility that MG modifies arginine residues in GPx. The results show that Arg 184 and Arg 185, located in the glutathione binding site of GPx was irreversively modified by treatment with MG. Reactive dicarbonyl compounds such as 3-deoxyglucosone, glyoxal and phenylglyoxal also inactivated GPx, although the rates for this inactivation varied widely. These data suggest that dicarbonyl compounds are able to directly inactivate GPx, resulting in an increase in intracellular peroxides which are responsible for oxidative cellular damage.

Keywords: Methylglyoxal; Glutathione peroxidase; Oxidative stress; MALDI-TOF-MS

INTRODUCTION

The Maillard reaction is a complex series of reactions between reducing sugars and protein amino groups, which lead to browning, fluorescence and protein cross-linking. Advanced glycation end products (AGEs) formed during the later stages of the Maillard reaction, accumulate in long lived tissue proteins, such as tissue collagens and lens crystallins, and may contribute to aging or the development diabetic complications and, atherosclerosis.[1,2] The dicarbonyl compounds Methylglyoxal (MG), 3-deoxyglucosone (3DG) and glyoxal (GO) have all been identified as intermediates in the Maillard reaction.[3]

MG, a reactive, α-dicarbonyl metabolite and physiological substrate for the glyoxalase system, is formed by non-enzymatic glycation and the enzymatic elimination of phosphate from dihydroxyacetone phosphate, glyceraldehyde-3-phosphate and by

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2003 Taylor & Francis Ltd
DOI: 10.1080/1071576021000041005

the oxidation of hydroxyacetone and aminoacetone.[4] The glyoxalase system uses reduced glutathione as a cofactor and catalyzes the conversion of MG to D-lactate via the intermediate *S*-D-lactoylglutathione. The formation of MG in cultured human red blood cells is increased under hyperglycemic conditions and by the addition of fructose D-glyceraldehyde, dihydroxyacetone, acetone and hydroxyacetone. The serum concentration of MG is increased by 5–6 fold in patients with insulin-dependent diabetes mellitus and 2–3 fold in patients with non-insulin-dependent diabetic mellitus.[5]

In a previous study, we reported that MG and 3DG induce heparin binding-epidermal growth factor (HB-EGF) in rat aortic smooth muscle cells (RASMCs) via the induction of oxidative stress.[6] Since HB-EGF is known to be a potent mitogen for smooth muscle cells and is abundant in atherosclerotic plaques, the induction of HB-EGF by MG and 3DG, as well as an incremental increase in intracellular peroxide levels may trigger atherogenesis during diabetes. However, the precise mechanism by which oxidative stress is caused by MG and 3DG remains unclear.

Reactive oxygen species (ROS) have been implicated in a wide range of biological functions, but they can be both essential and highly toxic to cellular homeostasis.[7] Under normal conditions, potentially toxic ROS are primarily generated via mitochondrial respiratory metabolism and are efficiently neutralized by cellular antioxidant defense mechanisms. However, several conditions are known to disturb the balance between the production of ROS and cellular defense, resulting in cellular destruction and dysfunction. An imbalance between pro- and anti-oxidant factors plays an important role in many disease processes, including diabetes mellitus.[8,9]

Among the anti-oxidant factors, antioxidant enzymes are generally thought to play an essential role scavenging ROS. Glutathione peroxidase (GPx), a potent anti-oxidative enzyme, scavenges a variety of peroxides. The overexpression of this enzyme was observed to suppress reactive oxygen-induced apoptosis in a variety of cells, suggesting that the inhibition of this enzyme is closely related to apoptotic cell death.[10] GPx contains a rare amino acid, selenocysteine (SECYS), which is essential for peroxidase activity. This SECYS residue resembles a cysteine residue in terms of its chemical properties but has a higher reactivity.[11] In a previous study, we showed that GPx was inactivated by NO by a mechanism in which SNAP, an NO donor, oxidizes Sec45 to form a mixed selenyl sulfide (Se–S) with a free thiol.[12] We also reported that NO induces HB-EGF via the inactivation of GPx and the subsequent induction of oxidative stress.[13] Hence, we hypothesized that MG also inhibits GPx activity, resulting in an increased oxidative stress.

In this study, we report that MG bound to GPx and suppressed its activity. We also determined the binding sites by means of MALDI-TOF-MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry).

MATERIALS AND METHODS

Materials

Bovine erythrocytes GPx was purchased from Sigma Co. Ltd. MG was purchased from Sigma and further purified by distillation under reduced pressure (b.p. 26°C, 20 mmHg) and the purity confirmed by NMR spectroscopy. The concentration of MG in stock solutions was determined by an end point enzymatic assay involving conversion to *S*-D-lactoylglutathione with glyoxalase I (Sigma) and hydrolysis catalyzed by glyoxalase II (Sigma).[14] GO and glutathione (GSH) were obtained from Sigma. *N*-(biotinoyl)-*N′*-(iodoacetyl) ethylenediamine (BIAM) were from Molecular Probes, Inc. Endopeptidase Lys-C were obtained from Boehringer Mannheim. Phenylglyoxal (PG) was obtained from Fluka. TMB and Reacti-bind maleic anhydride activated plates were obtained from Pierce. Other chemicals were of the highest grade available.

GPx Activity Determination

GPx activity was determined according to the method described by Lawrence and Burk.[15] The decrease in the concentration of NADPH was followed spectrophotometrically at 340 nm. The reaction mixture consisted of 240 mU/ml of glutathione reductase, 0.25 mM GSH, 0.15 mM NADPH in 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA; a 10 μl sample was added to this mixture and allowed to equilibrate at 37°C for 3 min. The reaction was initiated by the addition of sufficient 0.25 mM *tert*–butyl hydroperoxide to adjust the final volume of the assay mixture to 1 ml.

Labeling of GPx with BIAM and the Detection of Peptides Derived from BIAM-labeled GPx

GPx incubated with or without 5 mM MG in 1 ml of 20 mM Hepes–NaOH (pH 7.5) containing 1 mM EDTA was reduced by 100 μM DTT. The reduced enzyme was dialyzed against 50 mM Tris–Cl (pH 6.5) containing 1 mM EDTA. The dialyzed protein was then incubated in the dark at room temperature for 10 min in 4 ml of 50 mM Tris–Cl (pH 6.5) containing 0.5% Triton X-100, 5% glycerol, 150 mM NaCl, 1 mM EDTA and 10 μM BIAM. The biotinylation reaction was terminated by the addition of iodoacetamide to a final concentration of 20 mM after

which, the pH of the reaction mixture was adjusted to 7.5. After 10 min, the mixture was dialyzed against 50 mM Tris–Cl (pH 8.0) containing 1 mM EDTA. Dialyzed BIAM-labeled GPx was incubated overnight at 37°C with 5 μg of endopeptidase Lys-C. The resulting digest was separated by HPLC on a C_4 column; the peptides were eluted with a linear gradient (2–80%, v/v) of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min over 90 min. Fractions were collected, and a portion of each was analyzed for BIAM-label peptides; the peptides were immobilized on maleic anhydride-activated microplate (Pierce) and the labeled peptide was detected with horseradish peroxidase-conjugated streptavidin and the peroxidase substrate TMB (Pierce), 3,3′,5,5′-tetrabezidine, the oxidation of which was monitored spectrophotometrically at 405 nm.

Modification of GPx with MG and Digestion with Endopeptidase Lys-C

GPx (0.2 mg) was equilibrated in 50 mM phosphate buffer, pH 7.4, before treatment with 5 mM MG in final volume 200 μl at 37°C. The rate of modification was monitored by GPx activity as the loss of activity of the GPx as a function of time. At 15% residual activity, the reaction was quenched and excess MG was removed by chromatography over PD-10 column (Pharmacia). The modified and unmodified enzymes were eluted in final volume of 0.5 ml and concentrated in a Microcon-10 ultrafiltration device (Amicon) before re-equilibration in 50 mM phosphate, 0, 1 mM EDTA. A control experiment was carried out simultaneously under the same conditions but in the absence of the MG. After equilibration in the appropriate buffer, both the MG-modified GPx and control samples were incubated with 10 μg of endopeptidase Lys-C in the presence of 1 M urea for 16 h at 37°C.

Mass Spectrometry and Amino Acid Sequencing

Negative ion MALDI-TOF-MS was performed using a Voyager-RP time-of-flight (TOF) mass spectrometer equipped with a delayed-extraction system (PerSeptive Biosystems, Flamingham, MA) as described previously.[12] Solutions (1 μl) containing the fractionated GPx peptides from the endopeptidase Lys-C digests were placed on the flat surface of a stainless steel plate and mixed with the same volume of matrix solution; the supernatant of a 33% acetonitrile solution saturated with α-cyano-4-hydroxy cinnamic acid. Ions were generated by irradiating the sample area with the output of a nitrogen laser (337 nm). For amino acid sequencing, selected peptides were purified using C_4 and C_{18} columns and subjected to Edman degradation using a model Precise 494 click sequencer (Perkins Elmer Life Sciences).

Reversed Phase HPLC Analysis

Peptides produced by the digestion of GPx with endopeptidase Lys-C were separated by RP-HPLC (reverse phase-high performance liquid chromatography) (Shimazu) at a flow rate of 1.0 ml using a 150 × 4.6 − mm I.D. Develosil 300 C4-HG-5 column monitored at A_{215}. After analyzing all the detectable peaks by MALDI-TOF-MS, peptides of interest were further purified by using a 150 × 4.6 − mm I.D. Develosil ODS-HG-5 column (Nomura chemical, Aichi, Japan) and purified peptides were characterized by MALDI-TOF-MS. A gradient system formed between solvent A (0.1% trifluoroacetic acid in 2% acetonitrile) and solvent B (0.1% trifluoroacetic acid in 80% acetonitrile) was used.

RESULTS

Inactivation of GPx by MG and other Carbonyl Compounds

In order to determine whether MG inactivates the activity of GPx *in vitro*, bovine GPx was treated with MG, and then subjected to a peroxidase activity assay using *tert*–butyl hydroperoxide as a substrate. GPx activity decreased after incubation with MG in a dose and time dependent manner (Fig. 1). The activity was reduced to 10% after incubation with 5 mM MG for 9 h. Since GPx contains Arg in its active site, the effect of phenylglyoxal, which is an arginine specific modification reagent,[16] on the activity of GPx was also examined. GPx activity was found to be

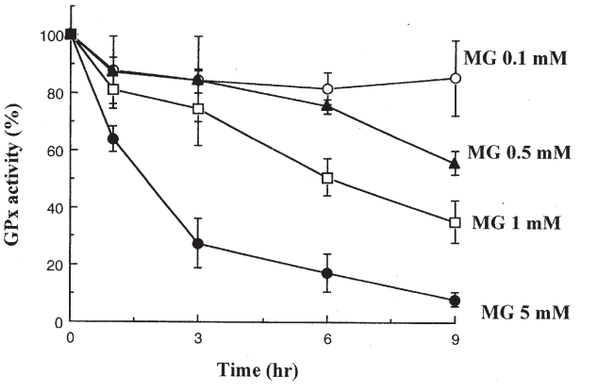

FIGURE 1 Inactivation of GPx by MG. GPx (0.2 mg/ml) was incubated with the indicated concentrations of MG in a reaction solution (50 mM phosphate buffer, pH 7.4, 1 mM EDTA) for the indicated times at 37°C. The GPx activity was analyzed as described in the "Materials and Methods" section. Activities are reported as the percentage of the control value.

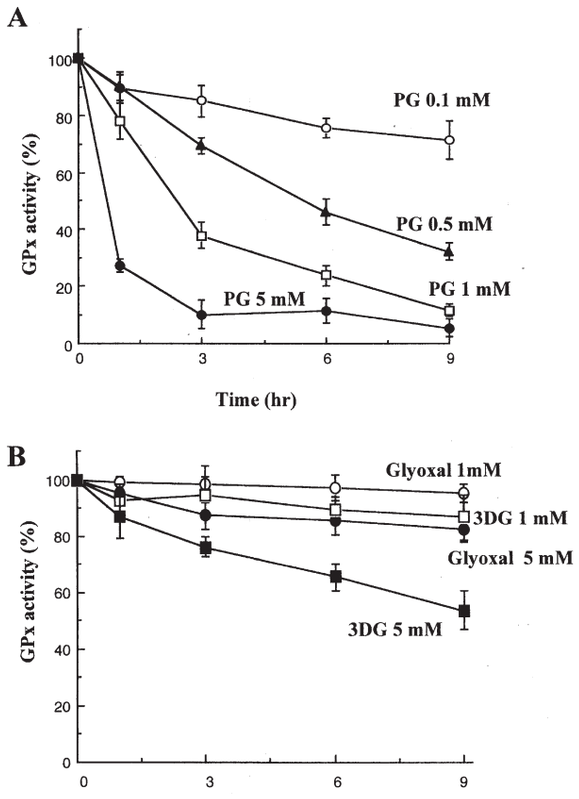

FIGURE 2 Inactivation of GPx by PG, 3DG, GO. (A) Effect of PG on GPx activity. (B) Effect of 3DG and GO on GPx activity. Conditions are the same as in Fig. 1.

inhibited by phenylglyoxal (Fig. 2A). The finding is similar to those observed with MG (Fig. 1). Similar studies were carried out with 3DG and GO, as shown in Fig. 2B. The rates of inactivation varied widely depending on the specific carbonyl compound used.

Detection of SECYS Derived from BIAM-labeled Peptide

GPx contains a penultimate SECYS residue at its active site that can be selectively labeled with an alkylating agent.[17] The reaction was carried out pH 6.5 to make more selective for SECYS. In low pH, selenol are more selective labeled than thiol group by BIAM, because of different pKa values for the ionization of a selenol (5.2) versus that of a sulfhydryl (8.3). To determine whether MG binds to such a residue, the purified protein was labeled with 10 μM BIAM at pH 6.5 and subsequently incubated with 2 mM idoacetamide at pH 7.5. The labeled protein was cleaved with endopeptidase Lys-C and BIAM-labeled peptide was detected using a maleic anhydride-activated microplate (Fig. 3). If MG binds to SECYS, BIAM-labeled peptides would be reduced in proportion to the extent of MG binding. However, no difference was detected between the control and MG treated sample (Fig. 3C). These data indicate that MG dose not bind to the SECYS residue at the active site of GPx.

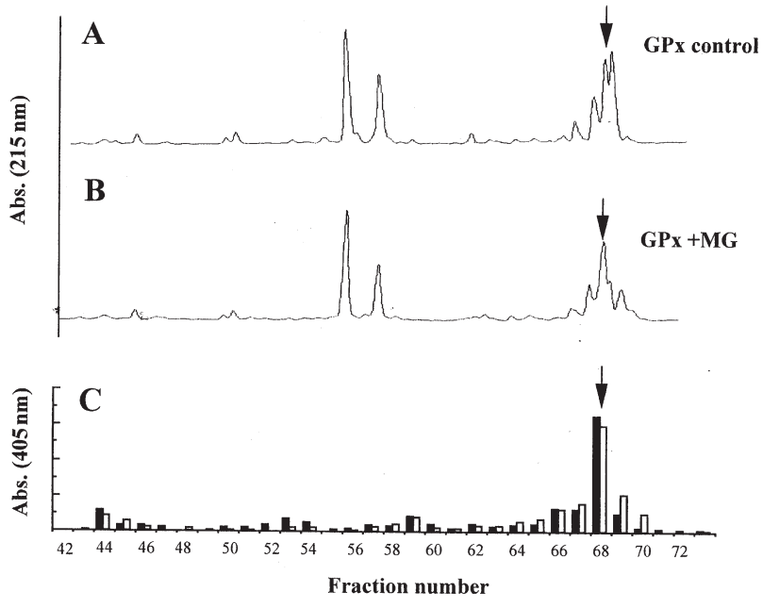

FIGURE 3 Detection of selenocysteine in peptides by BIAM-labeling (A) and (B). GPx was labeled with BIAM and digested with endopeptidase Lys-C, and the resulting peptides was analyzed on a C_4 column with elution monitored on the basis of the absorbance at 215 nm. (C) Each fraction from a C_4 column in A (close bar) and B (open bar) was analyzed for BIAM-labeled peptides. Peptides were immobilized on a maleic anhydride-activated microplate and the labeled peptide detected with horseradish peroxidase-conjugated streptavidin and the peroxidase substrate 3,3′,5,5′-tetramethyl benzidine, the oxidation of which was monitored spectrophotometrically at 405 nm.

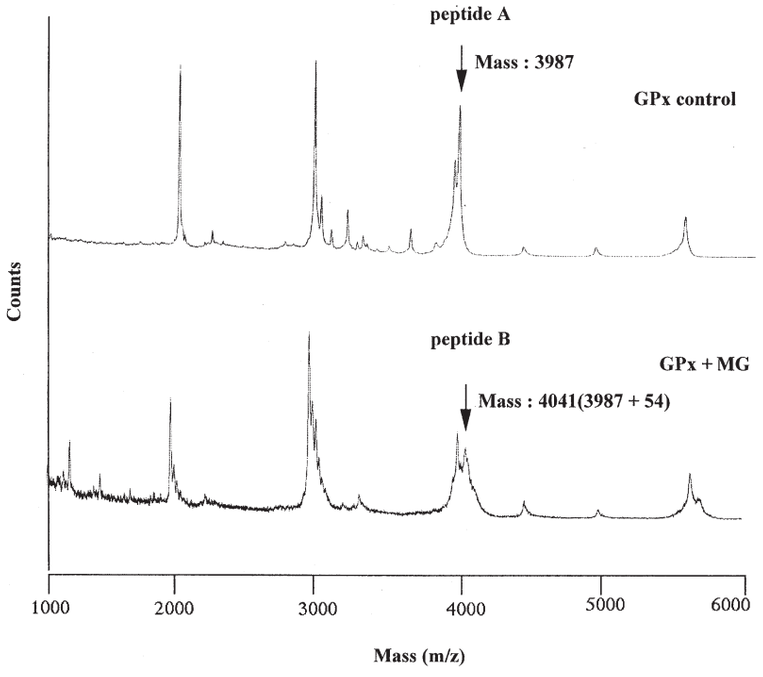

FIGURE 4 MALDI-TOF-MS analysis of endopeptidase Lys-C-digests of GPx with or without MG treatment. Endopeptidase Lys-C-digests of GPx treated with or without MG subjected to MALDI-TOF-MS for molecular mass measurement. The peptides indicated by arrows were denoted as peptide A (control) and B (MG-modified sample), respectively.

Determination of MG-binding Site

In order to determine the MG binding site, we performed MALDI-TOF-MS analysis and N-terminal sequencing. GPx was incubated with MG, followed by endopeptidase Lys-C treatment, and the resulting digest examined by MALDI-TOF-MS. The 3987 Da peak differs from the control by 54 Da (Fig. 4). Since the calculated molecular weight for MG added via modification is 54 Da, these data indicate that MG mainly binds to this peptide. The peaks indicated by arrows in Fig. 4 are denoted peptide A (control) and B (MG-modified sample), respectively. To further identify the MG-binding fragment, peptides A and B were purified using HPLC on C_4 and C_{18} columns and N-terminal sequencing was performed. Figure 5A shows the N-terminal sequencing profile. Arg 184 and 185 did not appear in the sequence of peptide B, indicating that MG bound to these two arginines. Furthermore, we also found that the same peptide was modified by treatment with phenylglyoxal (data not shown). These data suggest that Arg 184

A

GPx alone : F - L - V - G - P - D - G - V - P - V - R - R - Y - S - R* - R* peptide A

GPx + MG : F - L - V - G - P - D - G - V - P - V - R - R - Y - S - X - X peptide B

B

MCAAORSAAA LAAAAPRTVY AFSARPLAGG EPFNLSSLRG 40

KVLLIENVAS LSeGTTVR*DYT QMNDLQRRLG PRGLVVLGFP 80

CNQFGHQENA K*NEEILNCLK YVR*PGGGFEP NFMLFEKCEV 120

NGEKAHPLFA FLREVLPTPS DDATALMTDP KFITWSPVCR 160

NDVSWNFEKF LVGPDGVPVR RYS*R*RFLTID IEPDIETLLS 200

QGASA

FIGURE 5 Profile of protein sequences. Peptides A and B were purified using HPLC on C_4 and C_{18} columns as described in the "Materials and Methods" section and subjected to Edman degradation. (A) Sequences for peptide A and B. (B) Protein sequence of GPx. Underline indicates the location of peptides A and B. *indicates the GSH binding site on GPx.[36]

and 185 are readily modified by dicarbonyl compounds.

DISCUSSION

We previously reported that MG elevates intracellular ROS levels,[6] causing cell damage.[18] Intracellular ROS levels could be increased by MG in several ways. One is that ROS are produced during glycation reactions of amino acids or proteins with MG.[19] Another is that the glutathione content of cells could be depleted during MG metabolism by the glyoxalase system and the cells, therefore, would not be able to efficiently eliminate ROS. Another possible mechanism is that enzymes which scavenge ROS, such as GPx, glutathione reductase, superoxide dismutase and catalase[20,21] become modified by MG, since it is able to bind to and modify arginine, lysine and cysteine residues in proteins.[22,23] In this study, we found that GPx was inactivated by MG *in vitro* and determined that the amino acid residues modified were Arg 184 and 185, as evidenced by MALDI-TOF-MS and protein sequencing.

Hyperglycaemia is considered to be a major risk factor for diabetic vascular complications. In diabetic patients, hyperglycaemia induces an increase in non-enzymatically glycated products, which is believed to be a factor in the pathogenesis of diabetic complications. The non-enzymatic reaction between reducing sugars and proteins leads to the reversible formation of Schiff bases and Amadori products and ultimately to the irreversible formation of advanced glycation end products (AGEs). Several observations suggest dicarbonyl compounds such as MG, 3DG and GO, which are produced by the Maillard reaction play a role in the development of diabetic complications.[24]

Oxidative stress, resulting from an imbalance between ROS production and destruction by antioxidant scavenger systems, may also be involved in the pathogenesis of diabetic microangiopathy. In hyperglycemic conditions such as diabetes, ROS production may arise from glucose auto-oxidation, oxidative degradation of Amadori products and AGE–RAGE interactions.[25] If not completely scavenged by antioxidants, free radicals can lead to damage to DNA, proteins or lipids. Eukaryotic cells contain non enzymatic and enzymatic antioxidant defense systems such as GSH, Vitamins C and E and GPx, GR, SOD, and catalase for protection against oxidative damage.

MG has been shown to modify arginine residues[26] and inhibit a large number of enzymes such as GPx, GR and glyceraldehydes-3-phosphate dehydrogenase.[27] It has also been shown to alter the characteristics of proteins.[28] Moreover, MG decreases protein-SH and reduces GSH levels. It has previously been shown that MG decrease GSH content in platelets.[29] Depletion of GSH leads to the complete inhibition of GPx activity, which is particularly effective in scavenging platelet derived H_2O_2. Therefore, the decrease in GSH levels leads to the accumulation of hydrogen peroxide. It is likely that metabolic perturbations induced by MG such as GSH depletion and H_2O_2 accumulation would modify platelet function. In fact it has been shown that hydrogen peroxide is a potent inhibitor of platelet function. The findings herein show that MG directly inactivates GPx by modifying Arg 184 and Arg 185, which is located at the GSH binding site. These data indicate that MG elevates ROS not only via the reduction of GSH but also via the inhibition of GPx activity. Therefore, MG plays a role in the increase of intracellular oxidative stress. It is well known that increasing ROS levels induce apoptosis. Recent research indicates that the exposure of Jurkat cells to MG could lead to intense apoptosis by activating c-Jun N-terminal kinase (JNK) and apoptosis signal-regulating kinase 1(ASK-1) signal transduction pathway.[30,31] In addition MG synergistically enhances cisplatin-induced apoptosis through activation of protein kinase C δ (PKC δ) and PKC δ is critical to both cell death and cell survival pathways.[32]

In this study, we first determined the sites on a protein that are modified by MG by MALDI-TOF-MS with high selectivity and accuracy. Although the concentration of MG required to inactivate the GPx activity in these studies was in the mM level and appears to be higher than concentrations observed in diabetes; 5–10 μM. However, MG might slowly inactivates GPx activity especially in diabetes and the lengthy exposure of GPx to MG might cause modification and have effects on pathogenesis. In the process of this study, several groups reported that 3DG inhibits GPx activity *in vitro*,[33] and that MG also inhibits GPx activity in smooth muscle cells.[34] However, the mechanisms responsible for this inactivation remains unknown. In addition, a decreased GPx activity in plasma or erythrocytes has been reported in patients with diabetic retinopathy and cataracts.[25,35] The actual clinical significance of decreased GPx activity in tissues continues to remain uncertain. Further investigation will be needed to determine whether GPx is modified by MG in a diabetic environment.

In conclusion, the results of the present study suggests that MG plays a significant role in protein modification and suggests that it may contribute to diabetic complications via the inactivation of GPx resulting in an increase in ROS that are responsible for oxidative cellular damage.

Acknowledgements

We wish to thank our laboratory colleagues for helpful advice during the course of this study. This study was supported, in part, by Grants-in Aid from the Ministry of Education, Science, Sports and Culture, Japan. Y.S. Park is the recipient of a research fellowship from the Japan Society for the Promotion of Science for Young Scientists.

References

[1] Vlassara, H. (1994) "Recent progress on the biologic and clinical significance of advanced glycosylation end products", *J. Lab. Clin. Med.* **124**, 19–30.
[2] Baynes, J.W. and Thorpe, S.R. (2000) "Glycoxidation and lipoxidation in atherogenesis", *Free Radic. Biol. Med.* **28**, 1708–1716.
[3] Thornalley, P.J., Langborg, A. and Minhas, H.S. (1999) "Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose", *Biochem. J.* **344**, 109–116.
[4] Kalapos, M.P. (1999) "Methylglyoxal in living organisms: chemistry, biochemistry, toxicology and biological implications", *Toxicol. Lett.* **110**, 145–175.
[5] McLellan, A.C., Thornalley, P.J., Benn, J. and Sonksen, P.H. (1994) "Glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications", *Clin. Sci., (Lond.)* **87**, 21–29.
[6] Che, W., Asahi, M., Takahashi, M., Kaneto, H., Okado, A., Higashiyama, S. and Taniguchi, N. (1997) "Selective induction of heparin-binding epidermal growth factor-like growth factor by methylglyoxal and 3-deoxyglucosone in rat aortic smooth muscle cells. The involvement of reactive oxygen species formation and a possible implication for atherogenesis in diabetes", *J. Biol. Chem.* **272**, 18453–18459.
[7] Halliwell, B. and Gutteridge, J.M.C. (1999) Free Radicals in Biology and Medicines, 3rd Ed., (Clarendon Press, Oxford).
[8] Finkel, T. and Holbrook, N.J. (2000) "Oxidants, oxidative stress and the biology of ageing", *Nature* **408**, 239–247.
[9] Baynes, J.W. (1991) "Role of oxidative stress in development of complications in diabetes", *Diabetes* **40**, 405–412.
[10] Hockenbery, D.M., Oltvai, Z.N., Yin, X.M., Milliman, C.L. and Korsmeyer, S.J. (1993) "Bcl-2 functions in an antioxidant pathway to prevent apoptosis", *Cell* **75**, 241–251.
[11] Stadtman, T.C. (1980) "Selenium-dependent enzymes", *Annu. Rev. Biochem.* **49**, 93–110.
[12] Asahi, M., Fujii, J., Takao, T., Kuzuya, T., Hori, M., Shimonishi, Y. and Taniguchi, N. (1997) "The oxidation of selenocysteine is involved in the inactivation of glutathione peroxidase by nitric oxide donor", *J. Biol. Chem.* **272**, 19152–19157.
[13] Koh, Y.H., Suzuki, K., Che, W., Park, Y.S., Miyamoto, Y., Higashiyama, S. and Taniguchi, N. (2001) "Inactivation of glutathione peroxidase by NO leads to the accumulation of H2O2 and the induction of HB-EGF via c-Jun NH_2-terminal kinase in rat aortic smooth muscle cells", *FASEB J.* **15**, 1472–1474.
[14] McLellan, A.C., Phillips, S.A. and Thornalley, P.J. (1992) "The assay of methylglyoxal in biological systems by derivatization with 1,2-diamino-4,5-dimethoxybenzene", *Anal. Biochem.* **206**, 17–23.
[15] Lawrence, R.A. and Burk, R.F. (1976) "Glutathione peroxidase activity in selenium-deficient rat liver", *Biochem. Biophys. Res. Commun.* **71**, 952–958.
[16] Takahashi, K. (1968) "The reaction of phenylglyoxal with arginine residues in proteins", *J. Biol. Chem.* **243**, 6171–6179.
[17] Tamura, T. and Stadtman, T.C. (1996) "A new selenoprotein from human lung adenocarcinoma cells: purification, properties, and thioredoxin reductase activity", *Proc. Natl Acad. Sci. USA* **93**, 1006–1011.
[18] Okado, A., Kawasaki, Y., Hasuike, Y., Takahashi, M., Teshima, T., Fujii, J. and Taniguchi, N. (1996) "Induction of apoptotic cell death by methylglyoxal and 3-deoxyglucosone in macrophage-derived cell lines", *Biochem. Biophys. Res. Commun.* **225**, 219–224.
[19] Yim, H.S., Kang, S.O., Hah, Y.C., Chock, P.B. and Yim, M.B. (1995) "Free radicals generated during the glycation reaction of amino acids by methylglyoxal. A model study of protein-cross-linked free radicals", *J. Biol. Chem.* **270**, 28228–28233.
[20] Vander Jagt, D.L., Hunsaker, L.A., Vander Jagt, T.J., Gomez, M.S., Gonzales, D.M., Deck, L.M. and Royer, R.E. (1997) "Inactivation of glutathione reductase by 4-hydroxynonenal and other endogenous aldehydes", *Biochem. Pharmacol.* **53**, 1133–1140.
[21] Choudhary, D., Chandra, D. and Kale, R.K. (1997) "Influence of methylglyoxal on antioxidant enzymes and oxidative damage", *Toxicol. Lett.* **93**, 141–152.
[22] Lo, T.W., Westwood, M.E., McLellan, A.C., Selwood, T. and Thornalley, P.J. (1994) "Binding and modification of proteins by methylglyoxal under physiological conditions. A kinetic and mechanistic study with *N* alpha-acetylarginine, *N* alpha-acetylcysteine, and *N* alpha-acetyllysine, and bovine serum albumin", *J. Biol. Chem.* **269**, 32299–32305.
[23] Uchida, K., Khor, O.T., Oya, T., Osawa, T., Yasuda, Y. and Miyata, T. (1997) "Protein modification by a Maillard reaction intermediate methylglyoxal. Immunochemical detection of fluorescent 5-methylimidazolone derivatives *in vivo*", *FEBS Lett.* **410**, 313–318.
[24] Yamada, H., Miyata, S., Igaki, N., Yatabe, H., Miyauchi, Y., Ohara, T., Sakai, M., Shoda, H., Oimomi, M. and Kasuga, M. (1994) "Increase in 3-deoxyglucosone levels in diabetic rat plasma. Specific *in vivo* determination of intermediate in advanced Maillard reaction", *J. Biol. Chem.* **269**, 20275–20280.
[25] Cekic, O., Bardak, Y., Totan, Y., Akyol, O. and Zilelioglu, G. (1999) "Superoxide dismutase, catalase, glutathione peroxidase and xanthine oxidase in diabetic rat lenses", *Ophthalmic Res.* **31**, 346–350.
[26] Oya, T., Hattori, N., Mizuno, Y., Miyata, S., Maeda, S., Osawa, T. and Uchida, K. (1999) "Methylglyoxal modification of protein. Chemical and immunochemical characterization of methylglyoxal-arginine adducts", *J. Biol. Chem.* **274**, 18492–18502.
[27] Ray, M., Basu, N. and Ray, S. (1997) "Inactivation of glyceraldehyde-3-phosphate dehydrogenase of human malignant cells by methylglyoxal", *Mol. Cell. Biochem.* **177**, 21–26.
[28] Riley, M.L. and Harding, J.J. (1995) "The reaction of methylglyoxal with human and bovine lens proteins", *Biochim. Biophys. Acta* **1270**, 36–43.
[29] Leoncini, G. and Poggi, M. (1996) "Effects of methylglyoxal on platelet hydrogen peroxide accumulation, aggregation and release reaction", *Cell Biochem. Funct.* **14**, 89–95.
[30] Du, J., Suzuki, H., Nagase, F., Akhand, A.A., Yokoyama, T., Miyata, T., Kurokawa, K. and Nakashima, I. (2000) "Methylglyoxal induces apoptosis in Jurkat leukemia T cells by activating c-Jun N-terminal kinase", *J. Cell Biochem.* **77**, 333–344.
[31] Du, J., Suzuki, H., Nagase, F., Akhand, A.A., Ma, X.Y., Yokoyama, T., Miyata, T., Kurokawa, K. and Nakashima, I. (2001) "Superoxide-mediated early oxidation and activation of ASK-1 are important for initiating methylglyoxal-induced apoptosis process", *Free Radic. Biol. Med.* **31**, 469–478.
[32] Godbout, J.P., Pesavento, J., Hartman, M.E., Manson, S.R. and Freund, G.G. (2002) "Methylglyoxal enhances cisplatin-induced cytotoxicity by activating protein kinase Cδ", *J. Biol. Chem.* **277**, 2554–2561.
[33] Niwa, T. and Tsukushi, S. (2001) "3-Deoxyglucoson-end AGEs in uremic complications: inactivation of glutathione peroxidase by 3-deoxyglucosone", *Kidney Int. Suppl.* **78**, S37–S41.
[34] Wu, L. and Juurlink, B.H. (2002) "Increased methylglyoxal and oxidative stress in hypertensive rat vascular smooth muscle cells", *Hypertension* **39**, 809–814.
[35] Kesavulu, M.M., Giri, R., Kameswara Rao, B. and Apparao, C. (2000) "Lipid peroxidation and antioxidant enzyme levels in type 2 diabetics with microvascular complications", *Diabetes Metab.* **26**, 387–392.
[36] Urshini, F., Maiorino, M., Brigelius-Flohe, R., Aumann, K.D., Roveri, A., Schomburg, D. and Flohe, L. (1995) "Diversity of Glutathione Peroxidase", *Methods Enzymol.* **252**, 38–53.