0273-2289/99/82/0167/$11.75

Protection of Myocardial Mitochondria Against Oxidative Damage by Selenium-Containing Abzyme m4G3

DAN-HUA QI,[1] GUI-MIN LUO,*[,1] LI ZHOU,[1] AND TONG-SHU YANG[2]

[1]National Laboratory of Enzyme Engineering, Jilin University, Changchun, 130023 China, E-mail: gmluo@mail.jlu.edu.cn; and [2]Institute of Epidemic Diseases, Bethune Medical University, Changchun, 130023 China

Received March 24, 1999; Revised June 26, 1999; Accepted July 27, 1999

Abstract

Selenium-containing abzyme (m4G3) was prepared and its protection of myocardial mitochondria against oxidative damage was studied using the swelling of mitochondria, quantity of lipid peroxidation products, and change in cytochrome-*c* oxidase activity as a measure of mitochondrial damage. The results showed that m4G3 could inhibit mitochondrial damage caused by the hypoxanthine–xanthine oxidase system in vitro. Electronic spin resonance (ESR) studies demonstrated that m4G3 could decrease the amount of free radicals generated in the damage system.

Index Entries: Abzyme m4G3; mitochondria; oxidative damage; xanthine oxidase/hypoxanthine system; glutathione peroxidase; enzyme mimics.

Introduction

Studies on radical medicine show that the initiation and development of many diseases, such as postischemic heart diseases, reperfusion of ischemic myocardium, brain ischemia, tumor, and various types of inflammation and physiological aging, relate to oxidation stress symptoms of radical metabolism disorder *(1)*. A series of a new type of antioxidation drugs, which are directed against the mechanism of inducing disease, was developed. These drugs can stop the injury caused by originally spontaneous free radicals or protect cells and tissues subjected to attacks of radicals from further injury. Glutathione peroxidase (GPX) is one of the most

*Author to whom all correspondence and reprint requests should be addressed.

important antioxidative enzymes. It can scavenge hydrogen peroxide and inhibit lipid peroxidation in vivo and prevent cells and tissues from lipid peroxidation, showing an aging-resistant effect *(2)*. Natural GPX does have some drawbacks, such as high molecular weight, poor stability, and limited availability, and scientists are making every effort to develop the artificial mimics of GPX. However, GPX mimics prepared so far display low activity and relative toxicity. We have prepared Se-containing abzyme m4A4, which possesses GPX activity, by chemical mutation *(3)*. In comparison with GPX, m4A4 shows many advantages *(4)*. Recently, we prepared an Se-abzyme m4G3 whose activity surpasses the activity of native GPX *(5)*. We consider Se-containing abzyme a potent antioxidant. For this reason, the antioxidation ability of abzyme m4G3 should be investigated. We demonstrated that m4G3 possesses strong antioxidation ability and has great application potential.

Materials and Methods

Materials

Hypoxanthine and thiobarbituric acid were obtained from a Shanghai second reagent plant. Cytochrome-*c* was obtained from a Tianjin biochemical plant. Glutathione (GSH), glutathione peroxidase (GPX), and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) were obtained from Sigma (St. Louis, MO). Tetraethoxypropane and HEPES were from Fluka (Buchs, Switzerland). Abzyme m4G3 was prepared according to ref. *5*. Xanthine oxidase and mitochondria were isolated and prepared from fresh milk and bovine heart, respectively. All other chemicals were of analytical grade.

Preparation of Mitochondria

Bovine heart mitochondria was isolated from fresh bovine heart according to ref. *6*. The separation medium was 150 m*M* KCl in 25 m*M* HEPES-NaOH buffer (pH 7.4). The concentration of the mitochondria protein was determined by the method of Commassie brilliant blue *(7)* using bovine serum albumin as the standard.

Preparation of Xanthine Oxidase

Xanthine oxidase was separated from fresh milk according to Ball's *(8)* method. Its specific activity was 0.3 U/mg.

Damage of Mitochondria and Antidamage System

A xanthine oxidase/hypoxanthine reaction system was used to generate free radicals. Xanthine oxidase oxidizes hypoxanthine to give active oxygen such as superoxide anion, H_2O_2, and hydroxy radicals, which initiate the chain reaction of lipid peroxidation *(9)*. All reagents were added into the reaction system according to Table 1. The control group included GSH in addition to mitochondria and buffer because GSH is an essential substrate for GPX, but it has an effect on lipid peroxidation *(10)*.

Table 1
Xanthine Oxidase/Hypoxanthine Reaction System (25°C)

Reactant	Control group	Damage group	Protection group
Hypoxanthine (0.2 m*M*)	–	+	+
Xanthine oxidase (0.01 U/mL)	–	+	+
EDTA (0.3 m*M*)	–	+	+
$FeSO_4$ (0.1 m*M*)	–	+	+
HEPES-NaOH buffer (25 m*M*, pH 7.4)	+	+	+
Mitochondria (2 mg/mL)	+	+	+
GSH (1 m*M*)	+	+	+
m4G3 (U/mL)	–	–	+

Abzyme m4G3 Assays

Abzyme m4G3 activity was measured as described by Wendel *(11)*. One unit of activity is defined as the amount of compound that utilizes 1 µmol of NADPH/min at 37°C.

Assays for Mitochondria Swelling

Swelling of mitochondria was assayed as described by Hunter et al. *(12)*. The various reagents were added according to Table 1, and swelling was measured as the decrease in turbidity of the reaction mixture at 520 nm. The decrease in the absorbance indicates an increase in the mitochondria swelling and a decrease in the mitochondria integrity.

Determination of Malondialdehyde

The level of lipid peroxidation was determined by the formation of malondialdehyde (MDA), the final product of lipid peroxidation. The various reagents were added according to Table 1 and allowed to react. Then the reaction was terminated by adding 0.1 mL of 0.5% (w/w) trichloroacetic acid. Finally, the MDA in mitochondria membrane was measured according to the thiobarbituric acid method *(13)* using tetraethoxypropane as the standard.

*Assay of Cytochrome-*c *Oxidase Activity*

The various reagents were added according to Table 1, and an aliquot was taken at different times and centrifuged (120,000*g*, 4°C, 10 min). The pellet was washed with 0.25 *M* sugar in HEPES-NaOH buffer (pH 7.4). Then it was suspended in a small amount of HEPES-NaOH buffer, and an aliquot was taken for assay of cytochrome-*c* oxidase (CCO) activity at 25°C in 2 mL of reaction system *(14)*, in which the cytochrome-*c* concentration was 15 µ*M*. First, 5 µL of 0.01 *M* $K_3Fe(CN)_6$ was added into the reference cell and allowed to oxidize the substrate completely; this was used as a control when time scanning was carried out. The absorbance was decreased with

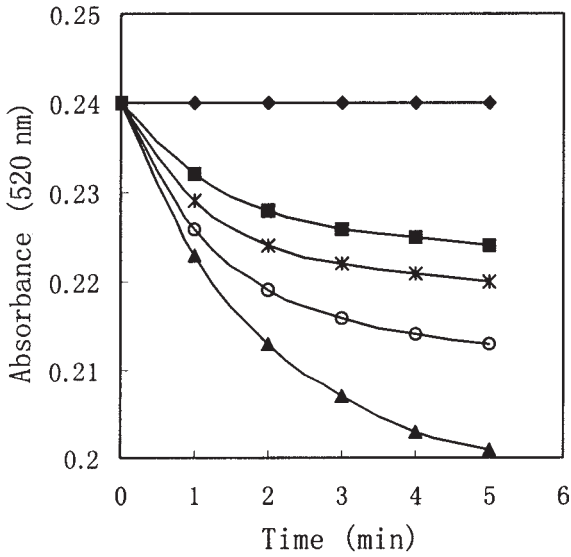

Fig. 1. Effect of abzyme m4G3 on swelling of mitochondria. (—◆—), Control; (—■—), damage + m4G3 (0.0063 U/mL); (—✳—), damage + m4G3 (0.00225 U/mL); (—○—), damage + m4G3 (0.00189 U/mL); (—▲—), damage.

oxidation of cytochrome-*c* in the sample cell, into which 5 µL of 0.01 *M* $K_3Fe(CN)_6$ was then added to oxidize cytochrome-*c* thoroughly when the reaction was complete. The absorbance at this time was denoted as A_∞. The plot of $\ln(A_t - A_\infty) \sim t$ was made. The absolute value of the line slope, K_{app}, was the apparent rate constant of cytochrome-*c* oxidation and was used to express the CCO activity. The specific activity was expressed as K_{app}/protein ($s^{-1}mg^{-1}$).

Measurement of ESR Spectra

The various reagents were added into a quartz capillary according to Table 1, and 20 m*M* DMPO was also added to use as a spin trap. ESR spectra were measured with a Bruker ER 200D EPR spectrometer *(15)*. The spectrometer settings were as follows: microwave frequency, 9.766 GHz; modulation frequency, 100 KHz; microwave power, 20.5 mW; scan speed, 1 G/s; magnify multiple, 1×10^6; central magnetic field, 3480 G; scan range, 100 G; and temperature, 25°C.

Results and Discussion

Effect of m4G3 on Swelling of Damaged Mitochondria

Figure 1 shows that the mitochondria were greatly swelled by the radical damage and that the mitochondria swelling was decreased by the addition of m4G3. The absorbance at 520 nm for the control group was basically constant, whereas the absorbance for the damage group decreased

Table 2
Effect of Abzyme m4G3
on Lipid Peroxidation of Mitochondria[a]

Concentration of $m4G_3$ (10^{-3} U/mL)	MDA (nmol/mg)	Relative content (%)
0 (control)	1.38	31.4
0 (damage)[b]	4.39	100
0.247	1.93	43.9
0.743	1.86	42.3
2.25	1.65	37.6
6.3	1.91	43.5
18.9	2.23	50.8
36.7	2.44	55.6

[a]All samples were damaged by the xanthine oxidase/hypoxanthine system for 1 h under the conditions described in the text except control.
[b]Content of MDA in the damage group is taken as 100%.

considerably with time, indicating that the mitochondria swelling was considerably increased. But the swelling for the protection group, which contained a certain concentration of m4G3, was apparently decreased, and the mitochondria swelling decreased with an increase in m4G3 concentration.

Inhibition of Lipid Peroxidation of Mitochondria by m4G3

The results showed that m4G3 could greatly inhibit lipid peroxidation of mitochondria and that the inhibition was considerably dependent on the m4G3 concentration (*see* Table 2). The amount of MDA decreased with an increase in m4G3 concentration. When the m4G3 concentration was 2.25×10^{-3}U/mL, the MDA content was only 37.6% of the damage group, indicating that 62.4% of MDA production was inhibited. When the m4G3 concentration was greater than 2.25×10^{-3}U/mL, the inhibition of m4G3 was observed. The major reason for this phenomenon should be studied further.

Protection of CCO Activity in Damaged Mitochondria by m4G3

CCO is one of the important redox enzymes in cells and is also one of the marker enzymes of mitochondria. The integrity of mitochondrial membrane lipid is important for the display of enzyme activity. Table 3 shows that the protection of CCO increased with an increase in m4G3 concentration. When the m4G3 concentration was 2.25×10^{-3}U/mL, 87% of CCO activity was retained, whereas for the damage group only 47% of CCO activity was retained. However, when the m4G3 concentration was greater than 2.25×10^{-3}U/mL, the protection of CCO was decreased. The reason for this phenomenon is not clear.

Table 3
Effect of Abzyme m4G3
on CCO Activity in Damaged Mitochondria[a]

Concentration abzyme m4G3 (10^{-3} U/mL)	Special activity of CCO (U/mg)	Relative activity (%)
0 (control)[b]	43.2	100.0
0 (damage)	20.5	47.3
0.247	22.1	51.2
0.743	24.0	55.4
2.25	37.6	87.0
6.30	36.4	84.3
18.9	34.1	80.0
56.7	32.6	75.5

[a]All samples were damaged by the xanthine oxidase/hypoxanthine system for 1 h under the conditions described in the text except control.
[b]Activity of CCO in the control group is taken as 100%.

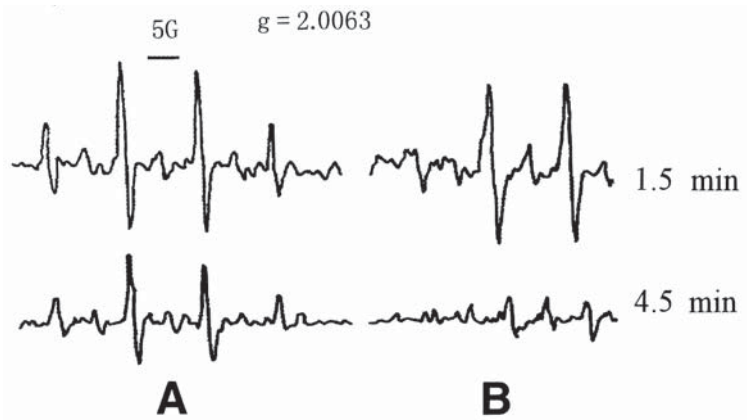

Fig. 2. Effect of abzyme m4G3 on the ESR spectra generated from xanthine oxidase/hypoxanthine damage system. **(A)** Xanthine oxidase/hypoxanthine damage system; **(B)** 1.75×10^{-3} U of abzyme m4G3. The spectra were recorded at 1.5 and 4.5 min after the addition of abzyme m4G3.

Quenching of the Radicals in the Xanthine Oxidase/Hypoxanthine System by m4G3

The 1:2:2:1 quartet shown in Fig. 2 is a typical signal of the adduct formed by DMPO and hydroxy free radical, indicating that hydroxy free radicals could be generated by the xanthine oxidase/hypoxanthine system, in which $O_2^{\cdot -}$ should be detected; however, only the signal of the DMPO-$OH^{\cdot}$ adduct could be detected in this experiment. This may be because of the presence of the dismutation reaction of $O_2^{\cdot -}$ catalyzed by the Fe^{2+}-EDTA complex. It is known that the radical number is proportional to the integral

of the area under the quartet. On the basis of the ratio between the area of damage (or protection) groups and the area of standard weak asphaltum (its spin number is 1.29×10^{13}), the spin numbers (or radical numbers) of the damage (or protection) groups could be calculated. For the damage group, the spin number was 2.0×10^{-14} at 1.5 min and 1.39×10^{-14} at 4.5 min. For the protection group, which contained 1.75×10^{-3} U/mL, the spin number was 1.87×10^{-14} at 1.5 min and 0.551×10^{-14} at 4.5 min. Thus, the addition of m4G3 could inhibit production of free radicals. Compared with the radical number of the damage group, the radical number of the protection group was decreased by 7% at 1.5 min and 70% at 4.5 min.

Mitochondria are known to be important cellular sites of both the production of reactive oxygen species and oxidative damage by these species. When mitochondria were attacked by free radicals, changes in composition, morphology, structure, integrity, and function took place. These changes were similar to what happens in cardiac diseases. We used xanthine oxidase/hypoxanthine, a free-radical generation system, to imitate the environment of the abnormal state in vivo and demonstrated that m4G3, as a radical scavenger, possessed a high antioxidative ability and can scavenge free radicals and prevent mitochondria from oxidative damage by active oxygen. This laid the experimental foundation for the application of abzyme m4G3 to medicine.

Acknowledgments

This work was supported by research grant 29171014 from NSF of China and grant 103-13-01-05 of "863" Projects in China.

References

1. Sies, H. (1993), *Free Rad. Biol. Med.* **14,** 313–323.
2. Chen, H., Pellett, L. J., and Andersen, H. J. (1993), *Free Rad. Biol. Med.* **14,** 473–482.
3. Luo, G. M., Zhu, Z. Q., Ding, L., Gao, G., Sun, Q. A., Liu, Z., Yang, T. S., and Shen, J. C. (1994), *Biochem. Biophys. Res. Commun.* **198,** 1240–1247.
4. Zhu, Z. Q., Ding, L., Luo, G. M., Liu, Z., Sun, Q. A., Yang, T. S., and Shen, J. C. (1994), *Biochem. Biophys. Res. Commun.* **202,** 1645–1650.
5. Luo, G. M., Ding, L., Liu, Z., Yang, T. S., and Ni, J. Z. (1998), *Ann. NY Acad. Sci.* **864,** 136–141.
6. Lansman, R. A., Shade, R. O., Shapiro, J. F., and Avise, J. C. (1981), *J. Mol. Evol.* **17,** 214–226.
7. Bradford, M. M. (1976), *Anal. Biochem.* **72,** 248.
8. Ball, E. G. (1939), *J. Biol. Chem.* **128,** 51–67.
9. Fong, K. L., McCay, P. B., and Poyer, J. L. (1973), *J. Biol. Chem.* **248,** 7792–7797.
10. Munday, R. and Winterbourn, C. C. (1989), *Biochem. Pharmacol.* **38,** 4349–4352.
11. Wendel, A. (1981), *Methods Enzymol.* **77,** 325.
12. Hunter, F. E., Scott, A., and Hoffsten, P. E. (1964), *J. Biol. Chem.* **239,** 614–621.
13. Mak, I. T., Misra, H. P., and Weglicki, W. B. (1983), *J. Biol. Chem.* **258,** 13,733–13,737.
14. Yonetani, T. (1961), *J. Biol. Chem.* **236,** 1680–1689.
15. Rashha-Step, J., Turro, N. J., and Cederbaum, A. I. (1993), *Arch. Biochem. Biophys.* **300,** 391–400.