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Short communication

Effects of γ-glutamylcysteine ethyl ester on heart mitochondrial creatine kinase activity: involvement of sulfhydryl groups

Hideharu Hayashi [a,*], Masaru Iimuro [b], Yuji Matsumoto [b], Masanori Kaneko [b]

[a] *Photon Medical Research Center, Hamamatsu University School of Medicine, 3600 Handa-cho, Hamamatsu 431-31, Japan*
[b] *Third Department of Internal Medicine, Hamamatsu University School of Medicine, 3600 Handa-cho, Hamamatsu 431-31, Japan*

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Abstract

To study the mechanism of the protective effect of γ-glutamylcysteine ethyl ester, mitochondrial creatine kinase activity of rat heart was measured. γ-Glutamylcysteine ethyl ester had a protective effect against the depression of creatine kinase activity induced by xanthine + xanthine oxidase or hydrogen peroxide. γ-Glutamylcysteine ethyl ester also prevented the depression of creatine kinase activity induced by *N*-ethylmaleimide. It is suggested that the protective effect of γ-glutamylcysteine ethyl ester is related to oxygen free radicals or to reduction of the sulfhydryl groups of the enzyme which were previously oxidized by oxygen free radicals.

Keywords: γ-Glutamylcysteine ethyl ester; Glutathione; Oxygen free radical; Mitochondrial creatine kinase; *N*-ethylmaleimide; Myocardial reperfusion injury

1. Introduction

Myocardial GSH is an important physiological antioxidant (Meister and Anderson, 1983). It has been shown that the decrease in tissue GSH level in ischemic/reperfused hearts (Ferrari et al., 1985) is linked with cellular dysfunction (Singh et al., 1989). It is, therefore, likely that the replenishment of GSH is important for the prevention of ischemia/reperfusion injury. However, GSH is not taken up by cells in its intact form and is degraded into its constituent amino acids before entering cells (Jensen and Meister, 1983).

γ-Glutamylcysteine ethyl ester, a precursor of GSH (Takimoto-Kamimura et al., 1990), can penetrate into cells and then be converted to GSH (Ohtsu et al., 1991). In fact, the administration of γ-glutamylcysteine ethyl ester in reperfused hearts has been shown to prevent the depletion of mitochondrial GSH and mitochondrial dysfunction (Nishinaka et al., 1991). In this regard, our question was whether γ-glutamylcysteine ethyl ester could prevent ischemia/reperfusion injury by increasing the GSH content.

We have reported that mitochondrial creatine kinase activity and the content of sulfhydryl groups were decreased by xanthine + xanthine oxidase or hydrogen peroxide, and that these changes did not occur when a sulfhydryl group reductant was added (Yuan et al., 1992). Since γ-glutamylcysteine ethyl ester contains sulfhydryl groups (Takimoto-Kamimura et al., 1990), it is possible that γ-glutamylcysteine ethyl ester shows protective effects by reacting with oxygen free radicals directly. Therefore, this study was undertaken to examine the direct protective effects of γ-glutamylcysteine ethyl ester against oxygen free radical-induced injury. To this end the activity of a mitochondrial membrane-bound enzyme (creatine kinase) was measured in vitro.

2. Materials and methods

2.1. Isolation of mitochondria and measurement of creatine kinase activity

Male Sprague–Dawley rats weighing 200–250 g were killed by decapitation. Hearts were rapidly excised, and the

* Corresponding author. Tel.: +81-53-435-2391; fax: +81-53-435-2391; e-mail: hide289@hama-med.ac.jp

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ventricular tissue was processed for the isolation of mitochondria by the method of Sordahl et al. (1971). Briefly, ventricles were homogenized in a Waring blender for 20 s in 10 volumes of 180 mM KCl, 10 mM EDTA, 0.5% bovine serum albumin (pH 7.4) (KEA medium). The homogenate was centrifuged at $1000 \times g$ for 10 min. The resultant supernatant was decanted through two layers of cheesecloth and further centrifuged at $10\,000 \times g$ for 20 min. The pellet was resuspended in KEA medium and centrifuged two more times at $10\,000 \times g$. The final pellet was suspended in 250 mM sucrose, 25 mM Tris–HCl (pH 7.0). The protein concentration was determined by the method of Lowry et al. (1951). Animal care and use were approved by the Local Animal Care and Use Committee of Hamamatsu University School of Medicine.

Creatine kinase activity was measured by the modified method of Hess et al. (1967), using creatine kinase assay kits (Wako Pure Chemical Industry). Briefly, 0.5 ml of incubation medium containing 25 mM creatine phosphate, 1.1 mM ADP, 2.2 U/ml hexokinase, 0.8 mM NADP, 1.1 U/ml glucose-6-phosphate dehydrogenase, 20 mM D-glucose, 0.73 mM nitrotetrazolium blue, and 50 mM phosphate buffer (pH 6.4) was preincubated for 3 min at 37°C. The reaction was started by the addition of mitochondria (100 μg protein/ml) and terminated 10 min later with 5 ml of 0.1 M HCl. The absorbance at 560 nm was measured. Activity was expressed as International Unit (IU)/mg protein. One unit is defined as the amount of enzyme that transforms 1 μmol of the substrate (creatine phosphate and ADP) in 1 min at 30°C.

2.2. Free radical-generating system

Superoxide anion radicals were generated by the xanthine oxidase (Terochem Laboratories) reaction with xanthine as a substrate (Kaneko et al., 1989). Since xanthine + xanthine oxidase reduces mitochondrial creatine kinase activity in a dose-dependent manner (Yuan et al., 1992), 133 μM xanthine + 0.002 U/ml xanthine oxidase were used in this study. It should be mentioned that xanthine oxidase was pretreated with 0.4 mM phenylmethylsulfonyl fluoride to inhibit trypsin-like activity which is present in the commercial product as a contaminant. Phenylmethylsulfonyl fluoride was found to exert no effect on the creatine kinase activity being measured in this study. Since hydrogen peroxide, which is a reactive oxygen species (not free radical per se), also reduces mitochondrial creatine kinase activity in a dose-dependent manner (Yuan et al., 1992), 1 mM hydrogen peroxide was used in this study. The concentration of *N*-ethylmaleimide (2 μM) was based on previous information (Yuan et al., 1992).

2.3. Statistical analyses

Results are presented as means ± S.E. For statistical evaluation, multiple analysis of variance was carried out and Duncan's multiple-range test was used to determine differences between the means within the population. $P < 0.05$ was taken to reflect a significant difference.

3. Results

3.1. Effects of γ-glutamylcysteine ethyl ester on the depression of mitochondrial creatine kinase activity in the presence of xanthine + xanthine oxidase or hydrogen peroxide

Table 1 shows the dose-dependent effects of γ-glutamylcysteine ethyl ester on creatine kinase activity in the presence of xanthine + xanthine oxidase or hydrogen peroxide. Incubation with 133 μM xanthine + 0.002 U/ml xanthine oxidase for 10 min at 37°C significantly depressed creatine kinase activity. The protective effect of γ-glutamylcysteine ethyl ester on creatine kinase activity was studied by incubating mitochondria with γ-glutamylcysteine ethyl ester (0.05–5 mM) in the presence of xanthine + xanthine oxidase. The inhibition of creatine kinase activity by xanthine + xanthine oxidase was significantly prevented by γ-glutamylcysteine ethyl ester in a dose-dependent manner.

The 10-min incubation of mitochondria with xanthine (133 μM), xanthine oxidase (0.002 U/ml), or γ-glutamylcysteine ethyl ester (1 mM) alone did not affect creatine kinase activity (1.39 ± 0.02, 1.39 ± 0.03, 1.39 ± 0.04 IU/mg protein, respectively).

Table 1 also shows the effects of γ-glutamylcysteine ethyl ester on creatine kinase activity induced by hydrogen peroxide. Mitochondria were incubated with γ-glutamylcysteine ethyl ester (0.05–5 mM) in the presence of hydro-

Table 1
Dose-dependent effects of γ-glutamylcysteine ethyl ester (γGCE) on heart mitochondrial creatine kinase activity in the presence of xanthine + xanthine oxidase (X + XO) or hydrogen peroxide (H_2O_2)

Concentration of γGCE (mM)	Creatine kinase activity (IU/mg protein)		
	Control	X + XO	H_2O_2
0	1.40 ± 0.06	0.51 ± 0.04	0.95 ± 0.04
0.05		0.62 ± 0.05	0.99 ± 0.06
0.1		0.80 ± 0.06[a]	1.18 ± 0.05[a]
0.5		1.11 ± 0.05[a]	1.25 ± 0.06[a]
1		1.31 ± 0.07[a]	1.37 ± 0.05[a]
5		1.30 ± 0.06[a]	1.39 ± 0.07[a]

Concentration-dependent effects of γ-glutamylcysteine ethyl ester (γGCE) on heart mitochondrial creatine kinase activity in the absence (Control) or presence of 133 μM xanthine + 0.002 U/ml xanthine oxidase (X + XO), or 1 mM hydrogen peroxide (H_2O_2). Mitochondria were preincubated for 10 min at 37°C with various concentrations (0.05–5 mM) of γ-glutamylcysteine ethyl ester. Each value is a mean ± S.E. of seven different preparations for X + XO, and is a mean ± S.E. of five different preparations for H_2O_2.

[a] Significantly different from control values (without γ-glutamylcysteine ethyl ester) ($P < 0.01$).

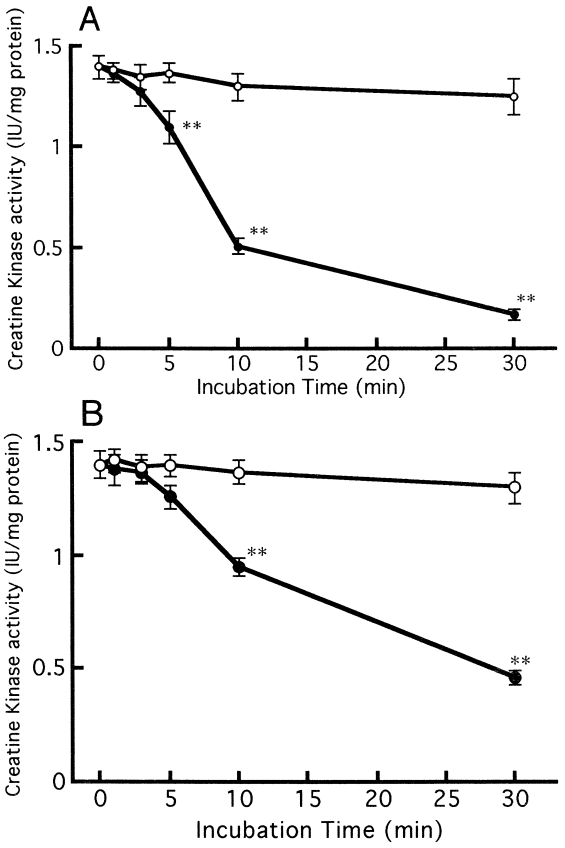

Fig. 1. Incubation time-dependent effects of γ-glutamylcysteine ethyl ester on the depression of mitochondrial creatine kinase activity in the presence of xanthine plus xanthine oxidase or hydrogen peroxide. (A) Effects of γ-glutamylcysteine ethyl ester in the presence of xanthine plus xanthine oxidase. Mitochondria were preincubated at 37°C with xanthine (133 μM) plus xanthine oxidase (0.002 U/ml). At indicated times of incubation, samples were assayed for creatine kinase activity. Each value is a mean ± S.E. of five different preparations. (●) Xanthine + xanthine oxidase; (○) Xanthine + xanthine oxidase in the presence of 1 mM γ-glutamylcysteine ethyl ester. ** Significantly different from control values ($P < 0.01$). (B) Effects of γ-glutamylcysteine ethyl ester in the presence of hydrogen peroxide. Mitochondria were preincubated at 37°C with hydrogen peroxide (1 mM). At indicated times of incubation, samples were assayed for creatine kinase activity. Each value is a mean ± S.E. of five different preparations. (●) Hydrogen peroxide; (○) Hydrogen peroxide in the presence of 1 mM γ-glutamylcysteine ethyl ester. ** Significantly different from control values ($P < 0.01$).

gen peroxide (1 mM). The inhibition of creatine kinase activity by hydrogen peroxide was significantly prevented by γ-glutamylcysteine ethyl ester in a dose-dependent manner.

Fig. 1A shows the protective effect of γ-glutamylcysteine ethyl ester on the incubation time-dependent inhibition of creatine kinase activity by xanthine + xanthine oxidase. The enzyme activity was measured after 1 to 30 min of preincubation with xanthine + xanthine oxidase in the presence and absence of γ-glutamylcysteine ethyl ester (1 mM). Significant inhibition of creatine kinase activity was observed after a 5-min preincubation with xanthine + xanthine oxidase. γ-Glutamylcysteine ethyl ester had a protective effect even after a 30-min preincubation with xanthine + xanthine oxidase.

Fig. 1B shows the protective effects of γ-glutamylcysteine ethyl ester on the incubation time-dependent inhibition of creatine kinase activity by hydrogen peroxide. The enzyme activity was measured after 1 to 30 min of preincubation with 1 mM hydrogen peroxide at 37°C in the presence and absence of γ-glutamylcysteine ethyl ester (1 mM). Significant inhibition of creatine kinase activity was observed after a 10-min preincubation with hydrogen peroxide. γ-Glutamylcysteine ethyl ester showed protective effects even after a 30-min preincubation with hydrogen peroxide.

3.2. Effects of γ-glutamylcysteine ethyl ester on the depression of creatine kinase activity by sulfhydryl reagent, N-ethylmaleimide

As shown in Fig. 2, mitochondrial creatine kinase activity was reduced by 50% after a 10-min incubation with 2 μM *N*-ethylmaleimide. Fig. 2 also shows the concentration-dependent effects of γ-glutamylcysteine ethyl ester on the depression of creatine kinase activity by *N*-ethylmaleimide. The inhibition of enzyme activity by *N*-ethylmaleimide was prevented by γ-glutamylcysteine ethyl ester in a dose-dependent manner.

When measured after 1 to 30 min of preincubation with 2 μM *N*-ethylmaleimide at 37°C, creatine kinase activity was inhibited after a 1-min preincubation. A high concentration (1 mM) of γ-glutamylcysteine ethyl ester had protective effects on enzyme activity even after a 30-min preincubation with *N*-ethylmaleimide (1.34 ± 0.06 IU/mg protein vs. 0.20 ± 0.04 IU/mg protein without γ-glutamylcysteine ethyl ester; $P < 0.01$).

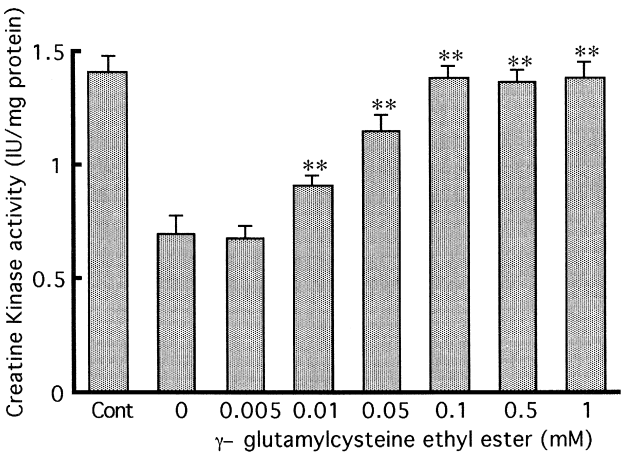

Fig. 2. Concentration-dependent effects of γ-glutamylcysteine ethyl ester on mitochondrial creatine kinase activity in the absence (Cont) or presence of *N*-ethylmaleimide (2 μM). Mitochondria were preincubated for 10 min at 37°C with various concentrations (0.005–1 mM) of γ-glutamylcysteine ethyl ester. Each value is a mean ± S.E. of five different preparations. ** Significantly different from control values (without γ-glutamylcysteine ethyl ester) ($P < 0.01$).

4. Discussion

Mitochondrial creatine kinase activity is reduced in reperfused hearts and has been shown to be related to cardiac function (Bittl et al., 1985). The depression of mitochondrial creatine kinase activity could be caused by oxygen free radicals in the ischemic/reperfused heart. In the present study, it was shown that the γ-glutamylcysteine ethyl ester prevented the depression of mitochondrial creatine kinase activity due to oxidative stress.

Since the effects of xanthine + xanthine oxidase or hydrogen peroxide on mitochondrial creatine kinase are prevented by superoxide dismutase or catalase, respectively (Yuan et al., 1992), it is likely that the depression of mitochondrial kinase activity is mediated by the generation of activated oxygen. Although there is a possibility that γ-glutamylcysteine ethyl ester reacts with xanthine oxidase directly, a similar protective effect of γ-glutamylcysteine ethyl ester on the depression of creatine kinase produced by xanthine + xanthine oxidase or hydrogen peroxide suggests that the protective effects were related to activated oxygen.

Glutathione synthetase promotes the following reaction:

γ-glutamylcysteine ethyl ester + glycine + ATP
⇌ GSH + ADP + Pi

This glutathione synthetase exists in the cytosol of cells and the mitochondria do not contain the enzyme (Griffith and Meister, 1985). Since we used isolated mitochondria in this experiment, it is likely that the protective effects of γ-glutamylcysteine ethyl ester on oxygen free radical injury were direct effects of this compound.

Since mitochondrial creatine kinase activity was depressed by *N*-ethylmaleimide, and since the depression of creatine kinase activity by oxygen free radicals was prevented by sulfhydryl group reductants such as dithiothreitol or cysteine (Yuan et al., 1992), the sulfhydryl groups of the enzyme protein are an important regulator of enzyme activity. Recently, nitric oxide (NO) has been shown to inhibit creatine kinase activity by nitrosylation of sulfhydryl groups (Gross et al., 1996). It was shown in our study that γ-glutamylcysteine ethyl ester had a protective effect on the depression of creatine kinase activity caused by *N*-ethylmaleimide. Therefore, the mechanism of the direct protective effects of γ-glutamylcysteine ethyl ester could involve the reduction of sulfhydryl groups of the enzyme which were previously oxidized by oxygen free radicals. However, it is also possible that γ-glutamylcysteine ethyl ester reacts with *N*-ethylmaleimide directly.

Another possibility may be a direct reaction of γ-glutamylcysteine ethyl ester with oxygen free radicals. It is, however, difficult to determine whether γ-glutamylcysteine ethyl ester acted with oxygen free radicals directly or reduced the sulfhydryl groups of the enzyme which were previously oxidized by oxygen free radicals.

In conclusion, it was shown that γ-glutamylcysteine ethyl ester protected the depression of mitochondrial creatine kinase activity induced by activated oxygen. Although the effects of γ-glutamylcysteine ethyl ester were likely to be related to oxygen free radicals and to sulfhydryl groups, the exact mechanism should be studied further.

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