The cardioprotective effect of γ -glutamylcysteine ethyl ester during coronary reperfusion in canine hearts

Yasuto Nishinaka, *Shigehisa Kitahara, †Satoru Sugiyama, Mitsuhiro Yokota, Hidehiko Saito & ¹†Takayuki Ozawa

Departments of Internal Medicine and †Biomedical Chemistry, Faculty of Medicine, University of Nagoya, Tsuruma, Showa-ku, Nagoya 466, Japan and *Teijin Institute for Biomedical Research 4-3-2 Asahigaoka, Hino, Tokyo 191, Japan

- 1 The cardioprotective effect of γ -glutamylcysteine ethyl ester was investigated on ischaemia-reperfusion-induced myocardial damage in anaesthetized dogs.
- 2 Open chest anaesthetized dogs were divided into four groups: 2 h occlusion of the left anterior descending coronary artery (LAD); 2 h LAD occlusion followed by 1 h reperfusion; 2 h LAD occlusion followed by 1 h reperfusion with administration of γ -glutamylcysteine ethyl ester (10 mg kg^{-1} just before reperfusion); 2 h LAD occlusion followed by 1 h reperfusion with administration of GSH (the reduced form of glutathione, 10 mg kg^{-1} just before reperfusion).
- 3 After occlusion or reperfusion, heart mitochondria were prepared from the normal area and the occluded or the reperfused area, and mitochondrial function (rate of oxygen consumption in State III, and respiratory control index) was measured polarographically.
- 4 Mitochondrial GSH and GSSG (the oxidized form of glutathione) concentrations, and activities of glutathione peroxidase and glutathione reductase were measured.
- 5 Two h of LAD occlusion induced mitochondrial dysfunction with depletion of mitochondrial GSH concentration. One h of reperfusion after 2 h LAD occlusion induced significant mitochondrial dysfunction associated with a marked depletion of mitochondrial GSH concentration.
- 6 γ -Glutamylcysteine ethyl ester reduced mitochondrial dysfunction and depletion of mitochondrial GSH concentration after 2 h LAD occlusion and 1 h reperfusion. In contrast, GSH did not prevent depletion of mitochondrial GSH concentration and mitochondrial dysfunction after 2 h LAD occlusion followed by 1 h reperfusion.
- 7 The activities of glutathione peroxidase and glutathione reductase did not change significantly in each group.
- 8 One h of reperfusion after 2 h occlusion of LAD induced ventricular arrhythmias. γ -Glutamylcysteine ethyl ester markedly reduced the development of reperfusion arrhythmias, whilst GSH showed no protective effect.
- 9 γ -Glutamylcysteine ethyl ester maintained mitochondrial GSH concentration, prevented reperfusion myocardial damage, and reduced reperfusion arrhythmias.

Keywords: γ -Glutamylcysteine ethyl ester; glutathione; mitochondria; ischaemia; reperfusion; arrhythmia

Introduction

Management of acute myocardial infarction is a major concern of many physicians, because myocardial infarction is a life threatening disease and might often be a cause of chronic heart failure. To reduce the amount of necrotic myocardium might lead to prevention of heart failure (Braunwald, 1976). Several invasive procedures such as intracoronary thrombolysis, percutaneous coronary angioplasty, and coronary bypass surgery for patients with acute myocardial infarction are performed to salvage jeopardized myocardium and beneficial effects of these procedures have been confirmed (DeWood *et al.*, 1983; Kloner *et al.*, 1983). Nevertheless, restoration of coronary blood flow is not always beneficial to ischaemic myocardium and exacerbation of ischaemia-induced myocardial dysfunction, i.e., reperfusion injury, is also recognized (Hearse, 1977).

Various pharmacological interventions have been proposed to prevent reperfusion injury. Since the generation of oxygen free radicals has been implicated as a pathophysiological mechanism involved in the transition from reversible to irreversible myocardial damage (Gauduel & Duvelleroy, 1984; Peterson *et al.*, 1985; Romaschin *et al.*, 1987; Ferrari *et al.*, 1989) treatment using radical scavengers has attracted much interest.

It is widely accepted that the reduced form of glutathione (GSH) is one of the most important physiological antioxidants, working alone and in concert with glutathione peroxidase and glutathione reductase. Curello *et al.* (1985) reported that ischaemia-reperfusion decreased tissue GSH concentration and that this decrease might be linked with cellular dysfunction. Accordingly, it is expected that administration of GSH would alter favourably reperfusion injury. However, GSH itself as a medicament is not suitable because GSH is not taken up by cells in its intact form, and is degraded into its constituent amino acids before entering cells (Jensen & Meister, 1983). Recently, γ -glutamylcysteine ethyl ester, a precursor of glutathione biosynthesis, has been developed (Kamimura *et al.*, 1990). γ -Glutamylcysteine ethyl ester may penetrate into cells in its intact form and then be converted by glutathione synthetase resulting in an increase in intracellular GSH concentration (Ohtsu *et al.*, 1991). Accordingly, in this paper, we examined effects of γ -glutamylcysteine ethyl ester on mitochondrial function during coronary reperfusion and on reperfusion arrhythmias in relation to changes in mitochondrial GSH and GSSG concentrations.

Methods

Animal preparations

This study was performed in accordance with the guidelines for animal experimentation set by the University of Nagoya.

¹ Author for correspondence.

Fifty-two adult mongrel dogs of either sex weighing from 8 to 13 kg were anaesthetized with intraperitoneal sodium pentobarbitone (50 mg kg^{-1}). After endotracheal intubation, artificial ventilation was instituted with a Harvard ventilator (Igarashi Ika Kogyo, Tokyo, Japan). Lead II of the electrocardiogram was monitored continuously throughout the experiment with a VC-640G oscillographic recorder (Nihon Kohden Co., Ltd., Tokyo, Japan). The right femoral vein was dissected and cannulated for the administration of test drug and adequate hydrating fluid. The right femoral artery was dissected and cannulated for the recording of arterial blood pressure (AD-600; Nihon Kohden). The chest of each dog was opened with a left fourth or fifth intercostal incision, and the heart was suspended in a pericardial cradle. The left anterior descending coronary artery (LAD) was dissected free distal to the first major diagonal branch. A 3-0 silk suture was placed under the vessel, and polyethylene tubing was threaded over the suture. Occlusion was produced by ligating the vessel and the tubing together, and reperfusion was induced by cutting the suture and tubing at the same time. Reperfusion was shown by the immediate appearance of hyperaemia in the reperfused area.

Experimental protocol

The dogs were divided into four groups. In group 1 ($n = 9$), the LAD was occluded for 2 h. In group 2 ($n = 17$), the LAD was occluded for 2 h followed by 1 h of reperfusion. In group 3 ($n = 10$), the LAD was occluded for 2 h and γ -glutamylcysteine ethyl ester (10 mg kg^{-1}) was administered intravenously just before 1 h of reperfusion. In group 4 ($n = 16$), GSH (10 mg kg^{-1}) was administered intravenously just before 1 h of reperfusion following 2 h occlusion of the LAD.

Estimation of mitochondrial function

Heart mitochondria were prepared from the normal area and the occluded or the reperfused area by differential centrifugation according to the method of Hatefi *et al.* (1961), and the rate of oxygen consumption in State III (St. III O_2) and the respiratory control index (RCI) were measured. St. III O_2 was calculated from mitochondrial oxygen consumption in ng atoms ($1 \text{ mole } \text{O}_2 = 2 \text{ gram atoms } \text{O}_2$) of oxygen consumed mg^{-1} mitochondrial protein min^{-1} during State III respiration. RCI was taken as the ratio between the rate of oxygen consumption before and after the addition of adenosine diphosphate (ADP). Oxygen consumption in mitochondria was measured polarographically with an oxygen electrode and a closed cell (UC-12; Central Kagaku, Tokyo, Japan) as described previously (Sano *et al.*, 1990).

Estimation of mitochondrial GSH and GSSG concentrations

Mitochondrial total glutathione (GSH + GSSG) concentration was determined by the DTNB-glutathione reductase recycling method described by Tietze (1969) and mitochondrial GSSG concentration was determined according to the method of Griffith (1980). Heart mitochondria were prepared from the normal area and the occluded or the reperfused area as described above, and 0.5 ml of the prepared heart mitochondria was added 0.05 ml of sulphosalicylic acid (27.5% w/v) and agitated with a mechanical device. The sulphosalicylic acid supernatant solution was obtained after centrifuging at $10,000 g$ for 10 min and used for the glutathione assay. Mitochondrial GSH concentration was deduced by subtracting the GSSG concentration from the total glutathione concentration.

Estimation of mitochondrial glutathione peroxidase and glutathione reductase activities

Glutathione peroxidase was determined according to the method of continuous monitoring of GSSG formation

described by Floré & Günzler (1984) with minor modifications (Sugiyama *et al.*, 1989). Prepared mitochondria diluted with 0.05 M potassium phosphate buffer was used as the enzyme source. The activity of glutathione peroxidase was expressed in units mg^{-1} mitochondrial protein. The amount of glutathione peroxidase inducing a net GSH decrease to 10% of the initial concentration in 1 min at 37°C at pH 7.0 is defined as 1 unit. Glutathione reductase was determined at 30°C according to the method of Carlberg & Mannervik (1985) with minor modifications. Prepared mitochondria diluted with 0.05 M potassium phosphate buffer was used as the enzyme source. The activity of glutathione reductase was expressed in units mg^{-1} mitochondrial protein, where 1 unit was defined as the amount of enzyme that catalyzed the oxidation of $1 \mu\text{mol}$ of NADPH min^{-1} .

Estimation of haemodynamic data

Arterial blood pressure was measured immediately before (0 min) and every 30 min during LAD occlusion and reperfusion. The number of ventricular premature beats and the total duration of ventricular tachycardia, which was defined as more than three successive ventricular premature beats, was also measured during LAD occlusion and reperfusion. In order to avoid artifacts such as LAD ligation and ventricular premature beats, heart rate was calculated only by sinus beats immediately before (0 min) and every 30 min after LAD ligation.

Statistics

The significance of all results was determined by Dunnett's test of analysis of variance except for mortality, for which the chi-squared test was used. Probability (P) values of less than 0.05 were considered statistically significant. All data in this paper are expressed as mean $\pm$ s.d.

Results

Mortality

Of the 52 dogs that underwent coronary artery occlusion, 5 died (1/9 in group 1; 1/17 in group 2; 2/10 in group 3; 1/16 in group 4). These were excluded from the subsequent analysis. During reperfusion, 15 dogs died from ventricular fibrillation (8/16 in group 2; 7/15 in group 4). Mortality in groups 2 and 4 was thus significantly higher ($P < 0.05$) than that in group 3. Data for the remaining 32 dogs form the basis of this paper.

Comparison of mitochondrial function

Figures 1 and 2 show St. III O_2 and RCI prepared from both the normal area and the occluded or the reperfused area in each group, respectively. In group 1 (2 h occlusion) mitochondrial function from the occluded area decreased significantly compared with the normal area. In group 2 (2 h occlusion followed by 1 h reperfusion) more severe mitochondrial dysfunction was observed. Administration of γ -glutamylcysteine ethyl ester significantly lessened mitochondrial dysfunction induced by 1 h reperfusion following 2 h occlusion, and indices of mitochondrial function from the reperfused area were improved significantly compared with the occluded area in group 1. Administration of GSH did not maintain mitochondrial function, and a similar decrease in mitochondrial function was observed to that in group 2.

Comparison of mitochondrial GSH and GSSG concentrations and activities of glutathione peroxidase and glutathione reductase

Table 1 shows mitochondrial total glutathione (GSH + GSSG), GSH, and GSSG concentrations prepared from both the normal area and the occluded or the reperfused area in each group, respectively. Two hour occlusion (group 1)

Figure 1 The rate of oxygen consumption in State III (St. III O₂) in heart mitochondria from the normal area (open columns) and the occluded or the reperused area (hatched columns) in each group. Group 1 = 2 h occlusion; Group 2 = 2 h occlusion/1 h reperfusion; Group 3 = 2 h occlusion/1 h reperfusion with γ-glutamylcysteine ethyl ester administration; Group 4 = 2 h occlusion/1 h reperfusion with GSH administration.

* $P < 0.05$; ** $P < 0.01$ versus normal area of the corresponding group.

† $P < 0.05$; †† $P < 0.01$ versus occluded area of Group 1.

§ $P < 0.01$ versus reperused area of Group 2.

In each group, St. III O₂ of the occluded or the reperused area decreased significantly compared with the normal area of the corresponding group. However, in group 3, St. III O₂ of the reperused area was maintained significantly compared with the occluded or the reperused area in groups 1 and 2. Each column shows the mean and vertical bars indicate s.d.

decreased mitochondrial total glutathione and GSH concentrations in the occluded area. This decrease was accelerated by 1 h reperfusion (group 2). Administration of γ-glutamylcysteine ethyl ester lessened the depletion of mitochondrial total glutathione and GSH concentrations induced by 1 h reperfusion following 2 h occlusion, and no significant changes were observed in mitochondrial total glutathione and GSH concentrations between the normal area and the reperused area. On the contrary, administration of GSH itself did not maintain mitochondrial total glutathione and GSH concentrations, and significant decreases were observed similar to

Figure 2 The respiratory control index (RCI) in heart mitochondria from the normal area (open columns) and the occluded or the reperused area (hatched columns) in each group.

* $P < 0.01$ versus normal area of the corresponding group.

† $P < 0.05$; †† $P < 0.01$ versus occluded area of Group 1.

§ $P < 0.01$ versus reperused area of Group 2.

In each group, RCI of the occluded or the reperused area decreased significantly compared with the normal area of the corresponding group. However, in group 3, RCI of the reperused area was maintained significantly compared with the occluded or the reperused area in groups 1 and 2. Each column shows the mean and vertical bars indicate s.d.

Table 1 Mitochondrial total glutathione, reduced glutathione (GSH), oxidized glutathione (GSSG) concentrations in each experimental group (nmol mg⁻¹ protein)

	Normal area	Occluded or reperused area
Total glutathione		
Group 1	4.03 ± 0.57	3.26 ± 0.51*
Group 2	4.12 ± 0.40	2.53 ± 0.66**†
Group 3	4.06 ± 0.52	3.57 ± 0.46§
Group 4	3.94 ± 0.33	2.44 ± 0.57**††
GSH		
Group 1	3.59 ± 0.46	2.87 ± 0.40*
Group 2	3.67 ± 0.45	2.16 ± 0.64**†
Group 3	3.68 ± 0.54	3.12 ± 0.40§
Group 4	3.67 ± 0.37	2.17 ± 0.52**†
GSSG		
Group 1	0.44 ± 0.28	0.40 ± 0.17
Group 2	0.45 ± 0.14	0.37 ± 0.15
Group 3	0.38 ± 0.09	0.45 ± 0.15
Group 4	0.27 ± 0.08	0.27 ± 0.09

Values are mean ± s.d.

* $P < 0.05$; ** $P < 0.01$ versus normal area of the corresponding group.

† $P < 0.05$; †† $P < 0.01$ versus occluded area of Group 1.

§ $P < 0.01$ versus reperused area of Group 2.

In group 2, mitochondrial total glutathione and GSH concentrations of the reperused area decreased significantly compared with the occluded area in group 1. However, in group 3, they were maintained significantly compared with that in group 2.

No significant changes were observed in mitochondrial GSSG concentration among the normal areas and the occluded or the reperused areas in all groups.

those in group 2. Mitochondrial GSSG concentration did not change significantly among the normal areas and the occluded or the reperused areas in all groups. Table 2 shows activities of mitochondrial glutathione peroxidase and glutathione reductase. No significant changes were observed in activities of mitochondrial glutathione peroxidase and glutathione reductase among the normal areas and the occluded or the reperused areas in all groups.

Haemodynamic data

Figure 3 shows the time course of arterial blood pressure in each group. No significant difference in arterial blood pressure was seen among the groups. Tables 3 and 4 show the time

Figure 3 The time course of arterial blood pressure (systolic and diastolic) in each group. The time immediately before left anterior descending coronary artery (LAD) ligation is expressed as 0 min. (○) Group 1 (2 h occlusion); (●) group 2 (2 h occlusion/1 h reperfusion); (△) group 3 (2 h occlusion/1 h reperfusion with γ-glutamylcysteine ethyl ester administration); (▲) group 4 (2 h occlusion/1 h reperfusion with GSH administration). No significant difference in arterial blood pressure was observed among the groups. Each symbol represents the mean and vertical bars indicate s.d.

Table 2 Mitochondrial glutathione peroxidase and glutathione reductase activities in each experimental group (m units mg⁻¹ protein)

	Glutathione peroxidase		Glutathione reductase	
	Normal area	Occluded or reperfused area	Normal area	Occluded or reperfused area
Group 1	17.03 ± 3.18	15.40 ± 3.57	7.99 ± 1.83	7.00 ± 1.85
Group 2	15.40 ± 5.32	12.73 ± 3.18	7.46 ± 1.34	5.83 ± 1.24
Group 3	16.95 ± 3.87	15.93 ± 3.51	7.44 ± 1.52	6.28 ± 1.67
Group 4	14.82 ± 3.17	11.54 ± 2.78	6.60 ± 1.27	5.35 ± 1.20

Values are mean ± s.d.

No significant changes were observed in activities of mitochondrial glutathione peroxidase and glutathione reductase among the normal areas and the occluded or the reperfused areas in all groups.

course of heart rate, the number of ventricular premature beats and the total duration of ventricular tachycardia in each group. No significant difference was observed during occlusion among the groups. However, administration of γ -glutamylcysteine ethyl ester reduced the number of ventricular premature beats and the total duration of ventricular tachycardia during reperfusion compared with those in groups 2 and 4 without inducing significant difference in heart rate among the groups.

Discussion

Mechanisms of reperfusion injury are considered to be complex biological phenomena and a number of factors have been suggested to contribute to the myocardial damage associated with ischaemia and reperfusion. These include myocardial high energy phosphate depletion (Jennings *et al.*, 1981; Jennings & Reimer, 1983), intracellular calcium overload (Fleckenstein *et al.*, 1974; Schanne *et al.*, 1979; Clusin *et al.*, 1983), inflammatory response (Fantone & Ward, 1982), loss of ionic homeostasis (Corr & Witkowski, 1983), labilization of lysosomes (Ogawa *et al.*, 1989), catecholamine (Singal *et al.*, 1981; 1982), and phospholipase-mediated membrane injury (Nagai *et al.*, 1985).

It is well known that adenine nucleotide breakdown to hypoxanthine and xanthine proceeds in ischaemic conditions and that superoxide anions are generated by xanthine oxidase after reperfusion (McCord, 1985). Neutrophils are also a major source of oxygen free radicals, and neutrophil migra-

tion is observed in necrotic myocardium (Romson *et al.*, 1982). Hence, generation of oxygen free radicals is expected to be enhanced in the ischaemia-reperfusion process. On the other hand, a living cell is provided with various radical scavenging systems such as superoxide dismutase, catalase, and glutathione. Glutathione is ubiquitously distributed, and has a wide range of radical scavenging functions. That is, its antioxidant function includes the removal of hydroperoxides, lipid peroxides, myeloperoxidase products, and also includes the removal of superoxide anions. Accordingly, glutathione is one of the most powerful intracellular antioxidant defences. Mitochondria contain most of the intracellular glutathione. Oxygen free radicals are produced endogenously by the mitochondrial electron transport system, and mitochondrial glutathione together with glutathione peroxidase might play an important role in scavenging these free radicals. Kajiyama *et al.* (1987) found that ischaemia-reperfusion decreased mitochondrial GSH concentration. Since oxygen free radical scavenging action by the glutathione redox system depends on GSH concentration itself within the range of cellular concentration, deterioration of the radical scavenging system in mitochondria might result from reperfusion. Since ischaemia-reperfusion might accelerate oxygen free radical formation, mitochondria during reperfusion phase are highly vulnerable to oxygen free radicals. Because mitochondria occupy a crucial role in cellular energy metabolism, mitochondrial dysfunction is directly linked with cellular dysfunction. Accordingly, a decrease in mitochondrial GSH concentration might play an important role in ischaemia-reperfusion-induced myocardial damage.

Table 3 Time course of heart rate (beats min⁻¹) in each group

	0	30	60	90	120	150	180 (min)
Group 1	119 ± 16	112 ± 13	104 ± 11	97 ± 14	94 ± 10		
Group 2	125 ± 10	114 ± 14	109 ± 15	102 ± 15	96 ± 12	95 ± 14	93 ± 12
Group 3	121 ± 12	117 ± 9	109 ± 9	99 ± 8	95 ± 4	92 ± 6	90 ± 5
Group 4	124 ± 18	117 ± 18	106 ± 16	95 ± 20	91 ± 17	91 ± 10	88 ± 8

Values are mean ± s.d.

The time immediately before left anterior descending coronary artery (LAD) ligation is expressed as 0 min.

Table 4 Number of ventricular premature beats and total duration of ventricular tachycardia (s) during occlusion and reperfusion

	Ventricular premature beats		Ventricular tachycardia	
	Occlusion	Reperfusion	Occlusion	Reperfusion
Group 1	30 ± 31		3 ± 6	
Group 2	11 ± 15	1376 ± 956	0 ± 1	1029 ± 671
Group 3	40 ± 51	32 ± 30*	1 ± 2	3 ± 5*
Group 4	42 ± 43	1731 ± 1574	1 ± 2	983 ± 959

Values are mean ± s.d.

* $P < 0.01$ versus reperfusion period of Group 2.

In this paper, we confirmed that ischaemia-induced mitochondrial dysfunction was accelerated by reperfusion, and that mitochondrial GSH concentration decreased significantly during occlusion, this decrease being exacerbated by reperfusion. Administration of γ -glutamylcysteine ethyl ester maintained mitochondrial GSH concentration and prevented reperfusion-accelerated mitochondrial dysfunction. GSH, a hydrophilic compound, cannot penetrate through cell membranes, and GSH administered exogenously might not be effective as an intracellular antioxidant. In these experiments it was confirmed that administration of GSH itself did not maintain mitochondrial GSH concentration and did not mitigate mitochondrial dysfunction associated with reperfusion.

The mechanism by which the concentrations of mitochondrial GSH decrease has not been fully elucidated. Kurosawa *et al.* (1990) found that proton gradient is necessary to maintain GSH in the mitochondrial matrix. Mitochondria deteriorated by ischaemia could not maintain proton gradient, and GSH might be washed out by reperfusion. In spite of administration of γ -glutamylcysteine ethyl ester, mitochondrial GSH concentration did not increase significantly in the normal area. GSH regulates its own biosynthesis via feedback inhibition (Gander *et al.*, 1983). However, it is reported that mitochondria do not contain enzymes for glutathione synthesis (Griffith & Meister, 1985). Accordingly, GSH concentration in mitochondria might regulate its own transport to mitochondria.

Reperfusion arrhythmias are another concern associated with reperfusion, because reperfusion arrhythmias might be a

cause of sudden death. Since oxygen free radicals cause peroxidation of lipids in cell membrane resulting in alteration in the ionic permeability, oxygen free radicals are likely to be involved in reperfusion arrhythmias (Manning & Hearse, 1984). In the present paper, administration of γ -glutamylcysteine ethyl ester also mitigated markedly the development of reperfusion arrhythmias. No protective effects of GSH itself were observed in reperfusion arrhythmias. In previous studies (Sugiyama *et al.*, 1980; Hanaki *et al.*, 1989), a relationship between disturbances in energy metabolism and the development of ventricular arrhythmias was proposed. We would like to point out here that both ventricular arrhythmias and mitochondrial dysfunction were reduced by γ -glutamylcysteine ethyl ester.

Drugs such as propranolol (Shatney *et al.*, 1976) and thromboxane synthetase inhibitor (Toki *et al.*, 1988) reduce infarct size, and these drugs are also reported to reduce ischaemia-induced mitochondrial dysfunction (Nagai *et al.*, 1984; Ogawa *et al.*, 1988). Accordingly, γ -glutamylcysteine ethyl ester might contribute to the reduction of infarct size.

In conclusion γ -glutamylcysteine ethyl ester maintained mitochondrial GSH concentration, and reduced reperfusion-induced mitochondrial dysfunction. γ -Glutamylcysteine ethyl ester also reduced the development of reperfusion arrhythmias.

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