Oral pretreatment with a green tea polyphenol for cardioprotection against ischemia–reperfusion injury in an isolated rat heart model

Shigeki Yanagi, MD,^a Kazuaki Matsumura, PhD,^b Akira Marui, MD, PhD,^a Manabu Morishima, MD,^a Suong-Hyu Hyon, PhD,^b Tadashi Ikeda, MD, PhD,^a and Ryuzo Sakata, MD, PhD^a

Objective: Ischemia–reperfusion injury is among the most serious problems in cardiac surgery. Epigallocatechin-3-gallate, a major polyphenolic component of green tea, is thought to be cardioprotective through its antioxidant activities. We investigated cardioprotective effects of oral epigallocatechin-3-gallate pretreatment against ischemia–reperfusion injury in isolated rat hearts and considered possible underlying mechanisms.

Methods: Rats were given epigallocatechin-3-gallate solution orally at 0.1, 1, or 10 mmol/L ($n = 12$ per group) for 2 weeks; controls ($n = 12$) received tap water alone for 2 weeks. Subsequently, Langendorff-perfused hearts were subjected to global ischemia for 30 minutes, followed by 60 minutes of reperfusion.

Results: Recoveries at 60 minutes after reperfusion of left ventricular developed pressure and maximum positive and minimum negative first derivatives of left ventricular pressure were significantly higher in 1-mmol/L group than in 0.1-mmol/L ($P < .0001$), 10-mmol/L ($P < .05$), and control ($P < .0001$) groups. Oxidative stress after reperfusion, as reflected by 8-hydroxy-2'-deoxyguanosine index, was lower in 1-mmol/L group than in control ($P < .01$) and 0.1-mmol/L ($P < .05$) groups. Western blot analysis after reperfusion showed p38 activation and active caspase-3 expression to be lower in 1-mmol/L group than in control group ($P < .05$).

Conclusions: Oral pretreatment with epigallocatechin-3-gallate preserved cardiac function after ischemia–reperfusion, an effect that may involve its antioxidative, antiapoptotic properties, although a high dose did not lead to dramatic improvement in cardiac function. Oral epigallocatechin-3-gallate pretreatment may be a novel and simple cardioprotective method for preventing perioperative cardiac dysfunction in cardiac surgery. (*J Thorac Cardiovasc Surg* 2011;141:511-7)

Ischemia–reperfusion injury (IRI) remains among the most serious problems in cardiac surgery. Several mechanisms and mediators of IRI have been described.¹ Reactive oxygen species reportedly play an important role in the pathogenesis of myocardial IRI.^{2,3} Administrations of various free radical scavengers and exogenous antioxidants have been demonstrated to reduce myocardial IRI and improve cardiac function in animal models.^{4,5}

Epigallocatechin-3-gallate (EGCG) is well known to be the most abundant polyphenolic catechin in green tea. Green tea is safe, popular, and known for its antioxidant properties, mainly through scavenging of reactive oxygen species (eg, superoxide anion, hydrogen peroxide, and hydroxyl radical).⁶ Several studies have shown green tea consumption to have potential protective effects against cardiovascular disease,^{7,8} thought to be attributable to its antioxidant activities.⁹ Little is known, however, about the cardioprotective

effects of preoperative oral intake of green tea polyphenols against ischemia followed by reperfusion of the heart.

We previously demonstrated, with a Langendorff-perfused rat heart model, that several exogenous antioxidants may enhance recovery after myocardial IRI by diminishing oxidative stress.^{10,11} In this study, we evaluated the significance of the cardioprotective effects against myocardial IRI of oral pretreatment with a green tea polyphenol and examined its dose effects, as well as the possible underlying mechanisms, in an isolated rat heart model.

MATERIALS AND METHODS

Green Tea Polyphenol Solutions

Green tea extract in pure form of EGCG was purchased from DSM Nutritional Products Ltd (Basel, Switzerland). EGCG solutions at concentrations of 1 mmol/L, 0.1 mmol/L, and 10 mmol/L were made with drinking water.

Animals

Male 11-week-old Sprague–Dawley rats (350–450 g body weight) were used for this study. All animals in this study received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health, (www.nap.edu/catalog/5140.html).

Study Groups

The rats were randomly divided into 4 groups of 12 animals each. In the 0.1-mmol/L, 1-mmol/L, and 10-mmol/L EGCG groups, rats were allowed

From the Department of Cardiovascular Surgery,^a Kyoto University Graduate School of Medicine, and the Institute for Frontier Medical Sciences,^b Kyoto University, Kyoto, Japan.

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Address for reprints: Akira Marui, MD, PhD, Department of Cardiovascular Surgery, Kyoto University Graduate School of Medicine, Postal code 606-8507, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto, Japan (E-mail: marmaru@tb3.so-net.ne.jp). 0022-5223/\$36.00

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Abbreviations and Acronyms

8-OHdG	= 8-hydroxy-2'-deoxyguanosine
dp/dt	= first derivative of left ventricular pressure
EGCG	= epigallocatechin-3-gallate
HR	= heart rate
IRI	= ischemia-reperfusion injury
LV	= left ventricle
MAPK	= mitogen-activated protein kinase

to drink the respective EGCG solutions as their daily fluid intakes for 2 weeks. In the control group, rats drank regular water for 2 weeks. Fluid consumption was measured daily for each rat.

Measurement of Plasma EGCG Levels

A technique of liquid chromatography coupled with tandem mass spectrometry was used to measure EGCG in rat plasma as previously described.¹² Just before the Langendorff study, a blood sample was obtained from the inferior vena cava of each rat that had been given EGCG for 2 weeks. Samples were centrifuged at 3000 g for 10 minute. The resulting plasma (100 μ L) was mixed with 20 μ L of an internal standard (gallic acid, 0.6 μ g/mL) and 20 μ L buffer solution (2% ascorbic acid and 1-mol/L sodium acetate, pH 5.0). The mixture was then extracted with 100 μ L of a methanol and acetic acid mixture (100/5 volume/volume) and centrifuged for 15 minutes at 11750g. After centrifugation, 100 μ L of the clear supernatant were transferred to another centrifuge tube, dried under nitrogen, and reconstituted with 100 μ L of mobile phase A. An aliquot of the solution was directly injected onto the setup for liquid chromatography coupled with tandem mass spectrometry for analysis, and the plasma EGCG concentration was calculated.

Heart Isolation and Perfusion

Isolated hearts were perfused with a Langendorff apparatus as previously described.¹⁰ The rats were anesthetized by inhalation of diethyl ether and intraperitoneal injection of sodium pentobarbital (50 mg/kg). Anticoagulation was achieved with an intravenous injection of heparin (1000 IU/kg). Each heart was quickly excised, washed in ice-cold saline solution, and mounted on a nonrecirculating Langendorff apparatus. After cannulation of the aorta, the coronary circulation was quickly restarted at a constant pressure of 80 mmHg at 37°C. Krebs-Henseleit solution (sodium chloride 118 mmol/L, potassium chloride 4.7 mmol/L, magnesium sulfate 1.2 mmol/L, sodium hydrogen carbonate 25 mmol/L, monobasic potassium phosphate 1.2 mmol/L, calcium chloride 2.5 mmol/L, and glucose 11 mmol/L) bubbled with a mixture of 95% oxygen and 5% carbon dioxide was used for perfusion. For measurement of left ventricular (LV) function, a water-filled latex balloon was inserted through the left atrium into the LV after a pulmonary arteriotomy. The balloon pressure was adjusted to maintain LV end-diastolic pressure at 10 mm Hg. After a stabilization period of at least 20 minutes, the isolated heart was subjected to no-flow global ischemia at 37°C for 30 minutes, followed by 60 minutes of reperfusion. The LV pressure waveform was traced on a strip chart.

Evaluation of LV Function

To measure LV pressure, the latex balloon was connected by a fluid-filled polyethylene tube to a pressure transducer. After achievement of preischemic perfusion equilibrium, heart rate (HR), LV developed pressure, and positive and negative first derivatives of LV pressure (+dp/dt and -dp/dt, reflecting both systolic and diastolic function) were measured and recorded continu-

ously with LV diastolic pressure stabilized at 10 mm Hg. Coronary flow was measured by collecting coronary effluent buffer. LV function was assessed by calculating the percentage recovery of each parameter (eg, % recovery of HR = HR after 60 minutes of reperfusion/baseline HR $\times$ 100%).

Immunohistochemical Assay for Evaluation of Oxidative Stress

The level of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a major product of oxidative DNA modifications,¹³ is widely used as a marker of oxidative stress against DNA.^{10,14} Cardiac tissues were fixed overnight in paraformaldehyde phosphate buffer solution immediately after reperfusion and then dehydrated sequentially with 50% and 70% ethanol for 24 hours. For immunohistochemical analysis, the avidin-biotin complex method was carried out as previously described.¹⁴ Briefly, after deparaffinization of the specimens, appropriately diluted solutions of normal rabbit serum (Dako Japan Co, Ltd, Kyoto, Japan), mouse monoclonal antibody against 8-OHdG (Japan Institute for the Control of Aging, Fukuroi, Japan), biotin-labeled rabbit anti-mouse IgG serum (Dako), and avidin-biotin complex (Vector Laboratories, Inc, Burlingame, Calif) were sequentially applied. The substrate for alkaline phosphatase (black) was obtained from a vector.

Immunohistologic data (8-OHdG index) were quantified as follows¹⁵: 8-OHdG index = $\Sigma[(X - \text{threshold}) \times \text{area (pixels)}]/\text{total cell number}$, where X is the staining density indicated in gray scale with ImageJ (National Institutes of Health, <http://rsbweb.nih.gov/ij/>) and Photoshop (Adobe Systems Inc, San Jose, Calif) software.

Western Blotting Analysis for Apoptosis-Related Proteins

Western blotting was performed to identify apoptosis in the myocardium at 60 minutes after reperfusion, as previously described.¹⁶ Ventricular tissue samples obtained from ischemic, reperfused hearts (n = 5 per group) were homogenized on ice in a lysis buffer with protease inhibitor and 1-mmol/L orthovanadic acid and quantified for protein levels with a commercially available assay (BCA protein assay reagent kit; Thermo Fisher Scientific Inc, Rockford, Ill). Proteins (20 μ g/sample) were separated with sodium dodecylsulfate polyacrylamide gels (20%) and electrotransferred onto nitrocellulose membranes (Immobilon P; Millipore, Billerica, Mass). After blocking with 5% nonfat milk in tris(hydroxymethyl)aminomethane-buffered saline solution containing 0.1% polysorbate 20, membranes were incubated overnight with the following first antibodies: (1) p38 kinase (sc-7972) at a 1:1000 dilution, (2) phosphorylated p38 (sc-7973) at a 1:1000 dilution, (3) caspase-3 (C9598) at a 1:1000 dilution, and (4) activated caspase-3 (C8487) at a 1:1000 dilution. After incubation with these primary antibodies, horseradish peroxidase-conjugated secondary antibodies were added (1:4000 dilution) for 1 hour at room temperature. Protein bands were enhanced with a chemiluminescence Western blotting determination kit (ECL-Plus; Amersham Pharmacia, Little Chalfont, UK). The band intensity was quantified with imaging software (ImageJ version 1.3).

Statistical Analysis

All data were expressed as mean $\pm$ SD. Statistical analyses were performed with statistical software (StatView for Windows version 5.0; SAS Institute Inc, Cary, NC). Data were analyzed by Student *t* test or 1-factor analysis of variance. When results were significant by analysis of variance, differences between individual groups were estimated with the Bonferroni-Dunn post hoc test.

RESULTS

Plasma EGCG Levels in Rats

In all groups, each rat received on average 30 mL/day of EGCG solution or tap water. As the dose of EGCG administered rose, plasma EGCG levels correspondingly increased

TABLE 1. Baseline parameters before ischemia

Variable	Control group (n = 12)	Epigallocatechin-3-gallate groups		
		0.1 mmol/L (n = 12)	1 mmol/L (n = 12)	10 mmol/L (n = 12)
Body weight (g)	422.8 ± 27.9	423.8 ± 19.2	420.1 ± 21.9	428.1 ± 19.1
Heart weight (g)	1.57 ± 0.08	1.61 ± 0.17	1.60 ± 0.14	1.62 ± 0.12
Heart weight/body weight (%)	0.37 ± 0.02	0.37 ± 0.03	0.38 ± 0.03	0.37 ± 0.03
Heart rate (beats/min)	315.4 ± 24.5	312.4 ± 20.4	326.0 ± 32.2	324.5 ± 38.4
LVDP (mm Hg)	96.6 ± 16.4	95.6 ± 11.6	98.5 ± 14.2	98.4 ± 14.3
Maximum +dp/dt (mm Hg/s)	2996.0 ± 538.2	2512.8 ± 301.7	3069.1 ± 727.3	2910.5 ± 654.3
Minimum -dp/dt (mm Hg/s)	2569.0 ± 514.8	2243.6 ± 267.7	2526.0 ± 450.0	2370.4 ± 425.6
Coronary flow (mL/min)	14.9 ± 3.8	16.8 ± 3.4	15.1 ± 3.3	17.1 ± 4.8

All values are mean ± SD. LVDP, Left ventricular developed pressure; dp/dt, first derivative of left ventricular pressure.

to a greater extent in rats given EGCG solution (0.1, 1, and 10 mmol/L) as drinking fluid for 14 days. Plasma EGCG was significantly higher in the 10-mmol/L group than in the control group (92.7 ± 29.8 vs 0 ng/mL, $P < .0001$), 0.1-mmol/L group (92.7 ± 29.8 vs 0 ng/mL, $P < .0001$), and 1-mmol/L group (92.7 ± 29.8 vs 6.2 ± 2.9 ng/mL, $P < .0001$). Plasma EGCG was undetectable in the 0.1-mmol/L group, just as in the control group.

Baseline Measurement Before Ischemia (Table 1)

There were no significant differences in body or heart weights before the Langendorff study among the 4 groups. Moreover, baseline LV function in the Langendorff study did not differ significantly among the groups.

Cardiac Function After Reperfusion

LV function measurements at 60 minutes after reperfusion are shown in Table 2. The percentage recoveries of LV developed pressure (Figure 1, B), maximum +dp/dt (Figure 1, C), and minimum -dp/dt (Figure 1, D) after 60 minutes of reperfusion were significantly higher in the 1-mmol/L and 10-mmol/L groups than in the control group. Among EGCG groups, the percentage recoveries of LV developed pressure (Figure 1, B), maximum +dp/dt (Figure 1, C), and minimum -dp/dt (Figure 1, D) were significantly higher in the 1-mmol/L group than in the 0.1-mmol/L and 10-mmol/L groups. There were no significant differences in HR among the 4 groups (Figure 1, A). Among all cardiac parameters, recovery after reperfusion in the 0.1-mmol/L group was almost equal to that in the control group. There

were no significant differences in coronary flow at 60 minutes of reperfusion among the groups (Table 2).

Oxidative Stress on DNA

Representative myocardial images of 8-OHdG immunohistochemical staining are shown in Figure 2, A. In the hearts of the 1-mmol/L and 10-mmol/L group rats, there were fewer darkly stained nuclei than in the control and 0.1-mmol/L groups. As shown in Figure 2, B, the 8-OHdG index calculated from staining was significantly lower in the 1-mmol/L group than in the control group (98.2 ± 39.4 vs $244.5 \pm 105.0 \times 10^2$, $P < .01$) and 0.1-mmol/L (98.2 ± 39.4 vs $221.9 \pm 79.1 \times 10^2$, $P < .05$) groups. This index was also significantly lower in the 10-mmol/L group than in the control group (125.9 ± 83.2 vs $244.5 \pm 105.0 \times 10^2$, $P < .05$).

Expression of Apoptosis-Related Proteins

Western blotting analyses for p38 (Figure 3, A) and caspase-3 (Figure 3, B) showed lower expressions of phosphorylated p38 and active caspase-3 in the 1-mmol/L group than in the control group ($P < .05$). Phosphorylation of p38 and caspase-3 cleavage were also significantly lower in the 10-mmol/L group than in the control group ($P < .05$).

DISCUSSION

We found oral pretreatment with EGCG to attenuate myocardial IRI and to preserve LV function after reperfusion in a Langendorff-perfused rat heart model. The antioxidative and antiapoptotic properties of EGCG may be involved in this cardioprotective effect. In addition, oral intake of

TABLE 2. Left ventricular function measurements at 60 minutes after reperfusion

Variable	Control group (n = 12)	Epigallocatechin-3-gallate groups		
		0.1 mmol/L (n = 12)	1 mmol/L (n = 12)	10 mmol/L (n = 12)
Heart rate (beats/min)	311.0 ± 46.1	301.6 ± 26.1	309.9 ± 22.3	298.4 ± 28.3
LVDP (mm Hg)	61.0 ± 17.4	59.3 ± 7.9	83.9 ± 12.8*†‡	72.3 ± 10.2§
Maximum +dp/dt (mm Hg/s)	1920.7 ± 591.0	1637.7 ± 174.8	2648.6 ± 599.5*†‡	2262.0 ± 595.4
Minimum -dp/dt (mm Hg/s)	1589.2 ± 378.9	1403.1 ± 167.1	2033.1 ± 291.7*†‡	1745.0 ± 345.9
Coronary flow (mL/min)	10.5 ± 2.7	12.2 ± 1.9	13.0 ± 2.4	12.7 ± 3.3

All values are mean ± SD. LVDP, Left ventricular developed pressure; dp/dt, first derivative of left ventricular pressure. * $P < .01$ versus control. † $P < .01$ versus 0.1 mmol/L. ‡ $P < .05$ versus 10 mmol/L. § $P < .05$ versus control. || $P < .05$ versus 0.1 mmol/L.

FIGURE 1. Recovery of left ventricular function at 60 minutes of reperfusion as percentage before ischemia. A, Heart rate (HR) did not differ significantly among groups. B–D, Recoveries of left ventricular developed pressure (LVDP, B), maximum positive first derivative of left ventricular pressure (Max+dp/dt, C), and minimum negative first derivative of left ventricular pressure (Min−dp/dt, D) in 1-mmol/L and 10-mmol/L groups were higher than in control and 0.1-mmol/L groups. Recoveries of left ventricular developed pressure and maximum positive and minimum negative first derivatives of left ventricular pressure were higher in 1-mmol/L group than in 10-mmol/L group. All values shown as mean $\pm$ SD, n = 12 per group.

a high dose of EGCG did not dramatically improve cardiac function after ischemia–reperfusion.

EGCG and Antioxidative Capacity

This novel pretreatment with EGCG, putting the whole body into an antioxidation state before IRI, may serve as a quite reasonable preconditioning method. One of the major theories, supported by most experimental evidence, suggests that reactive oxygen species generation is responsible for postischemic contractile dysfunction.¹⁷ Furthermore, clinical studies have shown that reactive oxygen species generation starts before cardiac surgery and that the availabilities of protective antioxidants depend on preoperative plasma antioxidant status.¹⁸ In this experiment, oral pretreatment with EGCG for 2 weeks before the Langendorff study had no effects on the animals' baseline cardiac parameters. Under IRI conditions, however, LV function was better maintained in the group pretreated with 1 mmol/L EGCG. The potent

antioxidative capacity of green tea polyphenols could contribute to LV function recovery after reperfusion, as shown by the lower 8-OHdG indices in the 1-mmol/L and 10-mmol/L groups.

EGCG and Antiapoptotic Effects

Another mechanism possibly underlying the cardioprotective effects of EGCG is its antiapoptotic effect, exerted through oral pretreatment, which may be associated with the preservation of LV function. Oxidative stress is now considered to be a major contributor, serving as a trigger, to myocardial apoptosis. Oxidative stress–induced apoptosis and its prevention by antioxidants have therefore also been analyzed.¹⁹ With respect to catechins, several in vitro investigations have shown EGCG to modulate multiple signal transduction pathways of apoptosis, such as mitogen-activated protein kinases (MAPKs). Furthermore, several studies have found caspase-3 cleavage, a downstream

FIGURE 2. Oxidative stress on DNA in cardiomyocytes. A, Representative immunohistochemical analysis images for 8-hydroxy-2'-deoxyguanosine (8-OHdG) were obtained with specific monoclonal antibody. Stained nuclei are indicated by arrows. Bar represents 50 μ m. B, Quantification of oxidative stress on DNA, as indicated by 8-hydroxy-2'-deoxyguanosine index. All values shown as mean $\pm$ SD.

substrate in caspases known to play an important role in regulating apoptosis, to be blocked by EGCG. In particular, EGCG has been shown to reduce the levels of p38 MAPK phosphorylation,²⁰ and several studies have confirmed inhibition of p38 MAPK phosphorylation to decrease apoptosis and improve cardiac function after myocardial IRI.²¹ Consistent with these findings, our Western blotting analysis demonstrated EGCG to protect against myocardial apoptosis by blocking p38 MAPK phosphorylation and caspase-3 cleavage, indicating that oral pretreatment with EGCG preserved LV function possibly by inhibiting myocardial apoptosis.

Dose Effects of EGCG

In this study, oral pretreatment with a high dose of EGCG did not yield the most potent cardioprotective effects. The cardiac function recovery rates after reperfusion were actually lower in the 10-mmol/L group than in the 1-mmol/L group, despite the blood EGCG concentration remaining

higher in the 10-mmol/L group. The plasma EGCG levels of rats given 1-mmol/L EGCG for 2 weeks may have exceeded the saturation point for cardioprotective effects. This result may also, however, be related to some additional adverse effects of EGCG. One possible adverse effect is that EGCG at high concentrations produces an excess of nitric oxide through activation of endothelial nitric oxide synthase. If nitric oxide exceeds a certain level, it reacts with superoxide anion under postischemic reperfusion conditions and yields more toxic radicals, such as hydroxyl radical and peroxynitrite anion, as demonstrated in several studies.²² The toxic activity of these reaction products may exceed the scavenger activity of EGCG, possibly leading to the suppression of heart function. These results underscore the importance of establishing the optimal oral EGCG dose to maintain recovery of cardiac function and minimize surgical complications, in anticipation of clinical application of EGCG.

Optimal Delivery of EGCG

We advocate that EGCG should be administered orally as a preoperative measure. Recently, with respect to the safety of EGCG, excessively high concentrations have been shown to be cytotoxic and to trigger genotoxic events in mammalian cells.²³ Direct administration of EGCG to the myocardium, such as by intravenous or intracoronary injection, reportedly produces harmful side effects, particularly at high doses. Oral EGCG, however, even when given as a very high bolus dose, is safe in human beings, as shown by clinical trial.²⁴ In this study, we selected the 2-week oral protocol for administration of EGCG on the basis of a report on the bioavailability of green tea polyphenols in rodents during long-term green tea consumption in drinking fluid.²⁵ Plasma concentrations of EGCG were shown to gradually increase in the first 2 weeks and then reach a plateau in rats given EGCG in drinking fluid.²⁵ In considering clinical applications, we will need to establish a period of oral administration of EGCG before heart surgery, referring to several clinical studies to determine the safety and pharmacokinetics of green tea polyphenols with long-term oral administration of EGCG.²⁴

Study Limitations

This study has several limitations. First, the heart model used in our experiment may not fully reflect in vivo conditions because of the lack of blood components such as neutrophils, platelets, and blood-derived cytokines. Several studies have demonstrated that neutrophils play an important role in the pathogenesis of IRI. An in vivo study taking into account the influence of blood is therefore in progress. Second, the type of ischemia used in this experimental model was global no-flow ischemia without cardioplegia, which is unlike the type of ischemia used in human heart surgery. This type of ischemia was adopted for this model to facilitate

FIGURE 3. Western blotting analysis for apoptosis-related proteins. A, p38 mitogen-activated protein kinase (MAPK), as phosphorylated p38 (p-p38) and nonphosphorylated p38 (t-p38). B, Caspase-3. All values shown as mean $\pm$ SD.

the evaluation of the cardioprotective effect of EGCG alone, eliminating other cardioprotective factors as far as possible. In the future, further investigations are needed to clarify the cardioprotective effects of oral pretreatment with EGCG in a larger animal model of ischemia with cardioplegia and cardiopulmonary bypass, in view of its clinical application to human heart surgery. These issues will be addressed in future green tea polyphenol clinical trials. Third, in this study, we did not incorporate preischemic measurement of oxidative stress or apoptosis. Although the baseline cardiac function did not differ among the groups, we cannot rule out the possibility that preischemic oxidative stress and apoptosis were suppressed in the EGCG groups. This point needs further investigation. Fourth, the involvement of p38 MAPK expression in apoptosis is still controversial. We cannot definitely conclude that the cardioprotective effect of EGCG exhibited in this study is attributable to p38-mediated apoptosis suppression. We selected p38 for evaluation in this study because in several previous reports EGCG was shown to suppress apoptosis through inhibition of p38 activation under conditions of myocardial IRI.²⁰

CONCLUSIONS

Oral pretreatment with EGCG, a green tea polyphenol, attenuated myocardial IRI and enhanced cardiac function recovery after ischemia followed by reperfusion in an isolated rat heart model. The mechanisms of oxidative stress suppression and myocardial apoptosis suppression may be involved in the cardioprotective effects of EGCG. In addition, oral intake of a high dose of EGCG did not dramatically improve cardiac function after ischemia–reperfusion. EGCG pretreatment by oral intake could be a novel and simple pre-

conditioning cardioprotective method for preventing perioperative cardiac dysfunction in cardiac surgical patients.

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