The Protective Effect of Epigallocatechin-3 Gallate on Ischemia/Reperfusion Injury in Isolated Rat Hearts: An ex vivo Approach

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The aim of this study was to evaluate the preventive role of epigallocatechin-3 gallate (EGCG, a derivative of green tea) in ischemia/reperfusion (I/R) injury of isolated rat hearts. It has been suggested that EGCG has beneficial health effects, including prevention of cancer and heart disease, and it is also a potent antioxidant. Rat hearts were subjected to 20 min of normoxia, 20 min of zero-flow ischemia and then 50 min of reperfusion. EGCG was perfused 10 min before ischemia and during the whole reperfusion period. EGCG significantly increased left ventricular developed pressure (LVDP) and increased maximum positive and negative dP/dt (+/−dP/dtmax). EGCG also significantly increased the coronary flow (CF) at baseline before ischemia and at the onset of the reperfusion period. Moreover, EGCG decreased left ventricular end diastolic pressure (LVEDP). This study showed that lipid peroxidation was inhibited and Mn-SOD and catalase expressions were increased in the presence of EGCG. In addition, EGCG increased levels of Bcl-2, Mn-superoxide dismutase (SOD), and catalase expression and decreased levels of Bax and increased the ratio of Bcl-2/Bax in isolated rat hearts. Cleaved caspase-3 was decreased after EGCG treatment. EGCG markedly decreased the infarct size while attenuating the increase in lactate dehydrogenase (LDH) levels in the effluent. In summary, we suggest that EGCG has a protective effect on I/R-associated hemodynamic alteration and injury by acting as an antioxidant and anti-apoptotic agent in one.

Key Words: Epigallocatechin-3 gallate, Ischemia/reperfusion injury, Apoptosis, Antioxidant

INTRODUCTION

Cardiovascular diseases represent the most significant health risk because they are responsible for more than 50% of total mortality. Among them, ischemic heart disease is the leading cause of morbidity and mortality and, according to the World Health Organization, will be the main global cause of death by the year 2020 [1]. Ischemia/reperfusion (I/R) heart injury results from severe impairment of coronary blood supply and leads to irreversible metabolic and ultrastructural changes. Numerous basic studies have suggested that strategies such as the administration of cardioprotective drugs at the start of reperfusion can significantly reduce infarct size [2,3].

Oxidative stress is a condition in which reactive oxygen species (ROS) or free radicals are generated and exert a toxic effect on cells. The heart is one of the major organs affected by ROS [4]. Oxygen free radicals are generated in the heart after I/R injury, and free radical scavenging enzymes, such as superoxide dismutase (SOD), and catalase have a protective function against I/R injury [5,6]. SOD and catalase have been shown to reduce infarct size in a porcine myocardial occlusion-reperfusion model [7]. I/R injury induces apoptosis during the reperfusion period, and inhibition of apoptosis by regulation of the ratio of Bax and Bcl-2 may improve cardiac contractility and vascular function [8,9].

Green tea is the most widely consumed beverage in the world. Hundreds of millions of people drink green tea around the world and studies suggest that green tea has many health benefits. The flavonoids of tea have antioxidant effects via attenuation of the inflammatory process in atherosclerosis, reduction of thrombosis, promotion of normal endothelial function, and inhibition of the expression of cellular adhesion molecules [10]. The main catechins in green tea are epicatechin, epicatechin-3-gallate, epigallocatechin and epigallocatechin-3 gallate (EGCG). The EGCG derivative is one of the major components of green tea. Studies have shown that EGCG has potential health benefits including anti-oxidant, cancer chemotherapeutic, and anti-inflammatory effects.
and 5% CO₂ and kept at 37 °C. In order to assess contractile function, a latex balloon connected to a pressure transducer was in- flated and immediately retrogradely perfused on a Langendorff apparatus with Krebs-Henseleit (K-H) solution (in mM: 119 NaCl, 4.7 KCl, 1.25 CaCl₂, 1.24 MgSO₄, 20.1 NaHCO₃, 1.24 KH₂PO₄, and 11.2 glucose) at a constant pressure of 80 mmHg. Perfusate was bubbled with 95% O₂ and 5% CO₂ and kept at 37°C. In order to assess contractile function, a latex balloon connected to a pressure transducer lab (ML-820, AD Instruments Pvt. Ltd., Australia) was inserted into the left ventricular cavity via the left atrium. It has been reported that EGCG can protect cardiomyocytes from I/R-induced apoptosis by inhibiting activation of signal transducer and activator of transcription 1 (STAT-1) [16]. In this study, we sought to determine whether the EGCG derivative of green tea has protective antioxidant and anti-apoptotic effects through regulation of Bax and Bcl-2 protein expression levels and cleaved caspase-3 activity in isolated rat hearts.

**METHODS**

**Animals**

Male Sprague-Dawley rats weighing 250~280 g (Orientbio Inc., Seoungnam, Korea) were housed in a temperature-controlled room with a 12 : 12-h light-dark cycle. The animals were provided free access to standard laboratory chow [5L79 Purina Rat & Mouse Chow (18%), Charles River Laboratories Inc., Wilmington, MA, USA] and water. The Animal Care Committee of Chonbuk National University Laboratory Animal Center approved the study.

**Perfusion of the rat hearts**

Rats were sacrificed by cervical dislocation. Hearts were immediately excised and the ascending aorta was cannulated and immediately retrogradely perfused on a Langendorff apparatus with Krebs-Henseleit (K-H) solution (in mM: 119 NaCl, 4.7 KCl, 1.25 CaCl₂, 1.24 MgSO₄, 20.1 NaHCO₃, 1.24 KH₂PO₄, and 11.2 glucose) at a constant pressure of 80 mmHg. Perfusate was bubbled with 95% O₂ and 5% CO₂ and kept at 37°C. In order to assess contractile function, a latex balloon connected to a pressure transducer lab (ML-820, AD Instruments Pvt. Ltd., Australia) was inserted into the left ventricular cavity via the left atrium. The animals were provided free access to standard laboratory chow [5L79 Purina Rat & Mouse Chow (18%), Charles River Laboratories Inc., Wilmington, MA, USA] and water. The Animal Care Committee of Chonbuk National University Laboratory Animal Center approved the study.

**Experimental protocols**

Hearts were perfused for a total of 90 min or 160 min, consisting of a 20-min pre-ischemia period followed by 20 min of global ischemia and 50 min or 120 min of reperfusion at 37°C (Fig. 1). The hearts were divided into three experimental groups. In group 1, hearts were continuously perfused with K-H buffer for 90 min without ischemia [sham, n=6]. In group 2, hearts were perfused with K-H buffer for a 20 min pre-ischemia period followed by 20 min of global ischemia and 50 min reperfusion at 37°C [control, n=12]. In group 3, hearts were perfused with 5 μM EGCG for 10 min before ischemia and during the 120 min reperfusion period, and group 2 also received this treatment [EGCG (5 μM), n=12]. At the end of the experiments, hearts were quick-frozen in liquid nitrogen and then stored at −70°C for Western blotting.

**Western blot analysis**

Total proteins were extracted from the left ventricle of the heart. The samples were placed in RIPA buffer [1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium de- cetyl sulfate (SDS), 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, 2 mM ethylenediaminetetraacetic acid] containing protease inhibitor, homogenized, incubated on ice for 1 h and then centrifuged at 13,400xg for 30 min. After determining the protein concentration of the supernatant in each sample in a modified Bradford assay, 50 μg of total protein was boiled in loading buffer for 5 min and loaded onto gradient SDS-polyacrylamide gels. Following electrophoresis, proteins were transferred to immobilon poly- vinylidene fluoride membranes. Membranes were blocked with TBS-T containing skim milk powder for 1 h at room temperature. The membranes were incubated with primary antibodies against Bax, Bcl-2, caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), catalase (Calbio- chem AG, San Diego, CA, USA), Cu/Zn-SOD, or Mn-SOD (Stressgen Biotechnologies, Victoria, BC, Canada). Proteins were detected after incubation with horseradish per- oxidase-conjugated secondary antibody for 1 h at room temperature. Immune reactivity was detected by chemiluminescence.

**Measurement of lactate dehydrogenase (LDH) in effluent**

The severity of myocardial injury was determined by measuring the concentration of LDH in the effluent. Effluents were collected just before ischemia and every 5 min during the 15-min reperfusion period in all groups, and the concentration of LDH was assayed using a LDH ELISA kit (Takara Bio Inc., Japan).

**Determination of myocardial infarct size**

Hearts were removed from the Langendorff apparatus after 120 min. Both the atria and root of the aorta were ex-
Ischemia/Reperfusion Injury and Epigallocatechin-3 Gallate

261

Fig. 2. Effect of EGCG on LVDP, LVEDP and ±dP/dt in isolated rat heart. EGCG was perfused 10 min before ischemia and during the whole reperfusion period. The percent changes of LVDP (A), LVEDP (B) and ±dP/dt (C, D) during reperfusion. Sham, non-ischemia; I/R, ischemia and reperfusion only; EGCG, ischemia and reperfusion treated with EGCG (5 μmol). I/R is square, EGCG is circle. *p < 0.01 vs. Sham group, p < 0.05 vs. I/R group.

cised and ventricles were frozen at −20°C for 1~2 h. Hearts were then sectioned (2- to 3-mm-thick), incubated in phosphate buffer (pH 7.4) containing 0.75% triphenyltetrazolium chloride (TTC) for 6 min at 37°C and fixed in 10% formalin. Infarcted areas were determined by planimetry (Analysis pro ver. 3.2, Soft Imaging System GmH, Germany). Infarct size was calculated as the percentage of the area-at-risk (% IS/AAR).

**Lipid peroxidation**

Lipid peroxidation was estimated from the formation of thiobarbituric acid reactive substances (TBARS). Cardiac tissue was homogenized in 6.5% trichloroacetic acid and a reagent containing 15% trichloroacetic acid, 0.375% TBA, and 0.25% HCl was added, mixed thoroughly, heated for 15 min in a boiling water bath, cooled, centrifuged, and the absorbance of the supernatant was measured at 535 nm against a blank that contained all the reagents except the tissue homogenate. Using malondialdehyde standard, TBARS were calculated as nanomoles per gram of wet tissue.

**Immunohistochemistry**

For fluorescence immunohistochemistry, hearts were frozen in liquid nitrogen and cut into 10-μm sections with a Microm HM525 cryostat (Microm, Walldorf, Germany) before fixing with 4% paraformaldehyde at room temperature for 1 h. Following washes, the preparations were blocked in serum-free protein block (Dako, Carpenteria, CA, USA) supplemented with 1% donkey serum for 1 h on a shaker table at room temperature. Sections were then incubated with rabbit anti-cleaved caspase-3 antibody (1 : 150; Chemicon International, Inc., Temecula, CA, USA) at 4°C overnight in Antibody Diluent (Dako, Carpenteria, CA, USA) supplemented with 0.5% donkey serum and 0.25% Triton X-100. The non-immune serum was used as a control. After complete washing, immunoreactivity was visualized with donkey anti-rabbit Alexa Fluor (488) (1 : 500; Invitrogen, Lidingo, Sweden) in a dark room. The section was subsequently counterstained with DAPI and mounted with fluorescent mounting medium (Dako, Carpenteria, CA, USA). Images were collected and analyzed using a Carl Zeiss Axiostop2 plus microscope (Carl Zeiss, Goettingen, Germany) and Axiovision 5.1 program. Photomicrographs were captured using a digital camera (Axioskop HRC, Carl Zeiss, Goettingen, Germany) with final magnification of 400×. The percentage of cells exhibiting cleaved caspase-3 immunoreactivity in the nucleus was calculated versus the total number of DAPI-stained cells using Image J software (ImageJ, U. S. National Institute of Health, Bethesda, MD, USA).

**Statistical analysis**

All values are presented as the mean±SEM unless otherwise specified. An unpaired t-test or analysis of variance (ANOVA) followed by Dunnett's method was used for the comparison of means between groups. p < 0.05 was considered statistically significant.
**RESULTS**

**Hemodynamic effect of EGCG in isolated rat hearts**

Hearts were perfused with EGCG 10 min before ischemia and during the whole reperfusion period (Fig. 1). Left ventricular developed pressure (LVDP) (Fig. 2A), LVEDP (Fig. 2B) and ±dP/dt (Fig. 2C, D) changed after 20 min of ischemia. Administration of EGCG increased LVDP and ±dP/dt and decreased LVEDP significantly during the reperfusion period compared to those in the control group.

**Effect of EGCG on coronary flow, LDH and infarct size in isolated rat hearts**

Coronary flow with EGCG changed both before and after 20 min of ischemia (Fig. 3A). EGCG increased the coronary flow significantly before ischemia and during the onset of the reperfusion period for about 10 min, compared to the control group. EGCG decreased the concentration of LDH in the effluent (Fig. 3B). After 120 min of reperfusion, the...
Ischemia/Reperfusion Injury and Epigallocatechin-3 Gallate

Effect of EGCG on lipid peroxidation and Cu/Zn-superoxide dismutase (SOD), Mn-SOD, and catalase protein expression in isolated rat hearts. After 50-min reperfusion, hearts were collected to measure lipid peroxidation (A), the protein expression. (B) The expression levels of Mn-SOD (C), Cu/Zn-SOD (D) and catalase (E) were determined by Western blotting. Results are representative of three independent experiments. Sham, non-ischemia; I/R, ischemia and reperfusion only; EGCG, ischemia and reperfusion treated with EGCG (5 μmol). *p < 0.05 vs. Sham group, #p < 0.05 vs. I/R group.

Effect of EGCG on lipid peroxidation, Mn-SOD, Cu/Zn-SOD and catalase in isolated rat hearts

In order to know the regulatory effect of EGCG against ROS, we first detected the lipid peroxidation. The lipid peroxidation ratio increased in I/R groups. The treatment of EGCG significantly inhibited the peroxidation (Fig. 6A). Catalase and superoxide dismutase are antioxidant enzymes that prevent free radical damage to the body. In order to observe whether EGCG had an antioxidant effect, we measured the expression of Cu/Zn-SOD, Mn-SOD and catalase proteins in isolated rat hearts. Mn-SOD, mitochondria-localized SOD, Cu/Zn-SOD, cytoplasm-localized SOD and catalase protein levels were assayed (Fig. 6B) and then each protein was densitometrically analyzed (Fig. 6C-E). EGCG significantly increased the expression of Mn-SOD and catalase compared to those in the I/R group. However, the expression of the Cu/Zn-SOD was not recovered by EGCG.

Effect of EGCG on cleaved caspase-3 in isolated rat hearts

Fig. 7A shows immunohistochemical staining of cleaved caspase-3, an important indicator of apoptotic cell death. The ratio of cleaved caspase-3 is shown in Fig. 7B. EGCG significantly decreased the ratio of cleaved caspase-3 compared to the control.

DISCUSSION

In this study, we administered EGCG 10 min before ischemia and during the whole reperfusion period. We found...
that administration of EGCG improved contractility during the reperfusion period by preventing apoptosis and via antioxidant effects in isolated rat hearts. EGCG decreased the expression of Bax protein and elevated that of Bcl-2 protein in isolated rat hearts compared to those in the control group. EGCG also increased cleaved caspase-3 activity and the levels of Mn-SOD and catalase proteins compared to those in the control group.

Our data revealed that EGCG increased coronary flow from baseline before global ischemia. Coronary flow increased from 12 mL/min (control) to 20 mL/min (EGCG). There was no significant difference in coronary flow during the posterior reperfusion period. However, during the onset of reperfusion, EGCG increased the coronary flow significantly. This finding indicates that EGCG itself had a vasodilating function, blocking vasoconstriction and thrombus formation in the coronary artery. EGCG prevents the development of atherosclerosis in apoE-deficient mice [17]. Acute actions of EGCG to stimulate production of nitric oxide from the endothelium may partially explain the beneficial effects of EGCG therapy in improving metabolic and cardiovascular pathophysiology in spontaneously hypertensive rats [18].

The early phase of reperfusion represents an important target for strategies to protect ischemic-reperfused myocardium [19]. Reperfusion injuries could be attenuated by intervention at the time of reperfusion [20]. When perfused before ischemia and during the whole reperfusion period, EGCG increased LVDP and ±dP/dt and decreased LVEDP significantly during the reperfusion period in isolated rat hearts. The recovery of LVDP with EGCG was about 40% while that without EGCG was about 20% after 50 min of reperfusion. In guinea pig hearts, EGCG protected against post-ischemic myocardial dysfunction and increased LVDP [21]. In another experiment, EGCG limited the infarct size via mitochondrial Kᵢₚₜ channel activation while isolated rat hearts showed no recovery of cardiac function [22]. However, the role of EGCG was interpreted as a regulator role similarly to ischemic precondition condition. The treatment schedule and concentration of EGCG were totally different from this study. Probably, the mitochondrial Kᵢₚₜ channel-associated pre-conditioning effect as well as the anti-oxidant effect is EGCG-induced protective roles against cardiac hemodynamic function alteration and associated damage.

EGCG may augment the metabolic and vascular actions of insulin. Therefore, It was also investigated whether the effects of EGCG treatment to simultaneously improve cardiovascular and metabolic function in spontaneously hypertensive rats (SHR; model of metabolic syndrome with hypertension, insulin resistance, and overweight). In acute studies, EGCG (1 ~ 100 μM) elicited dose-dependent vasodilation in mesenteric vascular beds (MVB) isolated from SHR ex vivo; this was inhibited by N(omega)-nitro-L-arginine methyl ester (L-NAME; nitric oxide synthase antagonist) or wortmannin [phosphatidylinositol (PI) 3-kinase inhibitor]. In chronic studies, 9-week-old SHR were treated by gavage for 3 wk with EGCG (200 mg/kg/day), enalapril (30 mg/kg/day), or vehicle. A separate group of SHR received L-NAME (80 mg/l in drinking water) for 3 wk with either EGCG or vehicle. Vasodilator actions of insulin were significantly improved in MVB from EGCG- or enalapril-treated SHR compared with vehicle-treated SHR. Both EGCG and enalapril therapy significantly lowered systolic blood pressure (SBP) in SHR. EGCG therapy of SHR significantly reduced infarct size and improved cardiac function in Langendorff-perfused hearts exposed to ischemia-reperfusion (I/R) injury. In SHR given L-NAME, beneficial effects of EGCG on SBP and I/R were not observed. Both enalapril and EGCG treatment of SHR improved insulin sensitivity and raised plasma adiponectin levels. We conclude that acute actions of EGCG to stimulate production of nitric oxide from endothelium using PI 3-kinase-dependent pathways may partially explain the beneficial effects of EGCG therapy to simultaneously improve metabolic and cardiovascular pathophysiology in SHR. These findings may be relevant to understanding the potential benefits of green tea consumption in patients with metabolic syndrome.

We found that acute administration of EGCG improved cardiac contractility during the reperfusion period. I/R injury is known to increase oxidants and oxygen radical formation and leads to subsequent leukocyte chemotaxis and inflammation [6]. Increased endogenous antioxidants could protect rat hearts from oxidative stress associated with ischemia/reperfusion injury [23]. The EGCG derivative of green tea is a potent antioxidant [24], EGCG scavenges free radicals induced by isoproterenol in Wistar rats pretreated with EGCG (30 mg/kg body weight) orally for 21 days [25]. In our study, levels of Mn-SOD and catalase expression were increased after I/R injury in the presence of EGCG in isolated rat hearts. Although Cu/Zn-SOD and Mn-SOD are both linked to the abrogation of O₂⁻, Cu/Zn-SOD is localized to cytoplasm, not mitochondria like Mn-SOD. Considering that ROS mainly originate from mitochondria, EGCG may have a specific regulatory role on the accumulation of mitochondrial O₂⁻, but less effect on cytoplasmic O₂⁻. Mitochondrial ROS and its associated mitochondrial damage is a determining factor in the loss of cardiomyocyte function and viability [26]. Similarly, overexpression of Mn-SOD, mitochondria-localized O₂⁻ inhibitor, in mice protects against I/R injury in vivo [27]. We found that EGCG protected against I/R injury in isolated rat hearts in part through an antioxidant effect.

I/R also induces cardiomyocyte apoptosis, and the inhibition of this apoptosis can prevent ischemia/reperfusion injury [28]. In vivo treatment with EGCG reduced ischemia/reperfusion injury by inhibiting the NF-κ B and AP-1 pathways in rat hearts [29]. EGCG also prevented I/R-induced apoptosis by inhibiting signal transducer and activator of transcription 1 (STAT-1) phosphorylation in isolated rat hearts [16]. Calcium/calmodulin-dependent protein kinase II inhibition prevented apoptosis by increasing the Bcl-2/Bax ratio and reducing caspase-3 activity in isolated cardiomyocytes [30]. Similarly, sulforaphane inhibited apoptosis by elevating the Bcl-2/Bax ratio and decreasing caspase-3 in isolated rat hearts [31]. Apoptosis is mediated by an activation cascade of the caspase family of cysteine proteases and ends with activation of the terminal effector caspase, caspase-3 [32]. Here we showed that EGCG increased the expression of Bcl-2 protein and decreased that of Bax protein in isolated rat hearts, in addition to decreasing cleaved caspase-3 activity. Our results showed that administration of EGCG in vitro could prevent apoptosis of cardiomyocytes by regulating pro-apoptotic and anti-apoptotic proteins such as Bax and Bcl-2, and by simultaneously regulating caspase-3 in isolated rat hearts.

I/R has frequently been applied to clarify the relationship between reactive oxygen species (ROS) and cell death. Although ROS are produced by several extracellular and
intracellular processes, in cardiomyocytes the mitochondria are the most relevant site for ROS formation [26]. Similarly, I/R-induced death is attenuated by over-expression of Mn-SOD or mitochondrial phospholipid hydroperoxide glutathione peroxidase (mito-PHGPx), but not by Cu, Zn-SOD, suggesting that mitochondrial ROS are one of the main causes of myocardial death. Considering that mitochondrial ROS, the Bcl-2/Bax ratio and the associated collapse of mitochondrial transmembrane potential (ΔΨm) are determining factors in ischemia-mediated myocardial damage [33,34], EGCG-induced cardiac protection mechanisms may include the inhibitions of mitochondrial ROS and its associated cell death mechanism, i.e., the increased ratio of Bcl-2 and BAX.

The limitation of this study is the clinical relevance of EGCG and its associated cell death mechanism, i.e., the increased ROS production against cardiac injury during I/R by preventing apoptosis via regulation of Bcl-2 and Bax expression and by antioxidant effects in isolated rat hearts.

ACKNOWLEDGEMENTS

This work was supported by the Korea Science and Engineering Foundation for the Biodos Research Program, Ministry of Sciences & Technology, and by the Korea Research Foundation (KRF-2009-0087202). This work is also supported by the National Research Foundation of Korea Grant (2009-0087202).

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