Polyphenol (-)-epigallocatechin gallate-induced cardioprotection may attenuate ischemia-reperfusion injury through adenosine receptor activation: a preliminary study

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Background: The activation of guanine nucleotide binding protein-coupled receptors, such as adenosine receptor (ADR) and opioid receptor (OPR), protects the heart against ischemia and reperfusion injury. We hypothesized that ADR or OPR might be involved in polyphenol (-)-epigallocatechin gallate (EGCG)-induced cardioprotection.

Methods: Langendorff perfused rat hearts were subjected to 30 min of regional ischemia and 2 h of reperfusion. Hearts were treated with 10 μM of EGCG, with or without the ADR or OPR antagonist at early reperfusion. Infarct size measured with 2,3,5-triphenyltetrazolium chloride staining was chosen as end-point.

Results: EGCG significantly reduced infarct volume as a percentage of ischemic volume (33.5 ± 4.1%) compared to control hearts (14.4 ± 1.1%, P < 0.001). A nonspecific ADR antagonist 8-(p-sulfophenyl) theophylline hydrate (27.1 ± 1.9%, P < 0.05 vs. EGCG) but not a nonspecific OPR antagonist naloxone (14.3 ± 1.3%, P > 0.05 vs. EGCG) blocked the anti-infarct effect by EGCG. The infarct reducing effect of EGCG was significantly reversed by 200 nM of the A₁ ADR antagonist DPCPX (25.9 ± 1.1%, P < 0.05) and 15 nM of the A₂B ADR antagonist MRS1706 (29.3 ± 1.7%, P < 0.01) but not by 10 μM of the A₂A ADR antagonist ZM241385 (23.9 ± 1.9%, P > 0.05 vs. EGCG) and 100 nM of the A₃ ADR antagonist MRS1334 (24.1 ± 1.8%, P > 0.05).

Conclusions: The infarct reducing effect of EGCG appears to involve activation of ADR, especially A₁ and A₂B ADR, but not OPR. (Korean J Anesthesiol 2012; 63: 340-345)

Key Words: Adenosine, Epigallocatechin gallate, Myocardial infarction, Reperfusion injury.
Introduction

Polyphenol (-)-epigallocatechin gallate (EGCG), a major catechin of green tea, by targeting ischemia [1] and reperfusion [2] provides cardioprotection against ischemia-reperfusion injury. We recently reported that the cardioprotective effect by EGCG was mediated via the ATP-sensitive potassium (K_{ATP}) channels [2].

On the other hand, homeostatic regulation and stress responses are mainly regulated by the extracellular signals transduced by guanine nucleotide binding protein (G-protein)-coupled receptor (GPCR) in the heart [3]. Adenosine receptor (ADR) and opioid receptor (OPR) belong to the GPCR family and activation of these upstream receptors might protect the heart by triggering second messengers [4,5].

We hypothesized that ADR or OPR might be activated by EGCG-induced cardioprotection. We therefore investigated the infarct reducing effect with ADR or OPR antagonists in EGCG-induced cardioprotection in isolated rat hearts.

Materials and Methods

The experimental procedures and protocols used in this study were reviewed and approved by our Institutional Animal Care and Use Committee.

Drugs and chemicals

EGCG, 8-(p-sulfophenyl)theophylline hydrate (8-SPT), and 2,3,5-triphenyltetrazolium chloride (TTC) were obtained from Sigma-Aldrich Chemical, St. Louis, MO, USA. Naloxone was purchased from Reyon Pharmaceutical Co., Seoul, Republic of Korea. Fluorescent polymer microspheres were purchased from Duke Scientific Co., Palo Alto, CA, USA. 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo [2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385), N-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]acetamide (MRS1706), and 1,4-dihydro-2-methyl-6-phenyl-4-(phenylethynyl)-3,5-pyridinedicarboxylic acid 3-ethyl-5-[(3-nitrophenyl)-methyl]ester (MRS1334) were purchased from Tocris Bioscience, Ellisville, MO, USA. Other chemicals were obtained from Sigma-Aldrich Chemical.

EGCG, 8-SPT and naloxone were dissolved in distilled water. DPCPX, ZM241385, MRS1706 and MRS1334 were dissolved in dimethyl sulfoxide. Stock chemicals were stored at −20°C and were diluted with Krebs-Henseleit (KH) solution to the required final concentrations on the day of each experiment.

Langendorff isolated heart perfusion preparation

Male Sprague-Dawley rats, weighing 280–330 gm obtained from KOATECH Co., Cheongwon-gun, Republic of Korea, were used. They received 50 mg/kg of pentobarbital sodium and 300 IU of heparin intraperitoneally. Hearts were isolated and perfused with modified KH solution containing (in mM) 118.5 NaCl, 4.7 KCl, 1.2 MgSO_4, 1.8 CaCl_2, 24.8 NaHCO_3, 1.2 K_HPO_4, and 10 glucose, as described previously [6]. Regional ischemia was induced by pulling the snare which was made at the level of the proximal length of the left coronary artery (LCA) and its major branches and confirmed by regional cyanosis and a substantial decrease in left ventricular developed pressure (LVDP). Reperfusion was started by releasing the snare.

Experimental protocol

All hearts were subjected to 30 min of regional ischemia and 120 min of reperfusion. Infusion of EGCG and antagonists was started 10 min before the onset of reperfusion and continued for 40 min (Fig. 1). To assess the involvement of ADR or OPR in EGCG-induced cardioprotection, ADR and OPR antagonists were perfused via 2nd port 10 min before EGCG perfusion. The concentrations of all chemicals were based on our and other previous studies on isolated working rat hearts that had no effect on infarct size in hearts subjected to ischemia and reperfusion [5,7-11].

Determination of area at risk and infarct size

At the end of each experiment, the LCA perfusion circuit was precluded, and diluted fluorescent polymer microspheres (Duke Scientific Corp., Palo Alto, MA, USA) were infused to demarcate the area at risk (AR). The hearts were cut into 2-mm
thick transverse slices using a rat heart slice matrix (Zivic Instruments, Pittsburgh, PA, USA). The slices were incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich Chemical, St. Louis, MO, USA) in sodium phosphate buffer (pH = 7.4) at 37°C for 20 min and subsequently immersed in 10% formalin to enhance the contrast. The left ventricle (LV) was removed from the remaining tissue. The myocardial AR in the LV was identified by illuminating with UV light. The necrotic area (AN, unstained with TTC) and AR (nonfluorescent under UV light) were traced on a clear acetate transparent sheet (Fig. 2B and 2C) and quantified with UTHSCSA Image Tool, version 3.0 (Department of Dental Diagnostic Science at The University of Texas Health Science Center, San Antonio, Texas, USA). The areas were converted into volumes by multiplying them by slice thickness. The AN volume was expressed as a percentage of the AR volume. All morphometric measurements were performed in a blinded fashion by a separate technician.

**Statistical analysis**

Data are presented as means ± SEM. Data analysis was performed with a personal computer statistical software package (SPSS for windows, Release 17.0; SPSS Inc, Chicago, IL, USA). Data were analyzed using one-way analysis of variance (ANOVA) with Tukey’s HSD post-hoc testing. Null hypotheses of no difference were rejected if P values were less than 0.05.

**Results**

A total of 96 rat hearts were used for infarct measurement experiment. Four rats were excluded during the stabilization period because of a CF > 18 ml/min or < 8 ml/min (2), LVDP < 80 mmHg (1), or HR < 250 beats/min (1). A further two hearts were excluded due to irreversible post-ventricular fibrillation pump failure (1 in control and 1 in EGCG + ZM241385). There-

![Figure 2](image-url)  
**Fig. 2.** (A) Regional ischemia was induced by pulling the snare at the level of the proximal left coronary artery, (B) The area at risk of left ventricle slice was identified by UV light illumination as the tissue without fluorescence. (C) The area of necrosis was identified by unstained area by TTC (closed circle) in area at risk.

| Group               | BW (gm) ± SEM | HW (gm) ± SEM | HW/BW (%) ± SEM | LV volume (cm³) ± SEM | AR volume (cm³) ± SEM | AR/LV (%) ± SEM |
|---------------------|---------------|---------------|-----------------|-----------------------|-----------------------|-----------------|
| CON                 | 315.6 ± 5.7   | 1.58 ± 0.03   | 0.50 ± 0.01     | 0.708 ± 0.027         | 0.492 ± 0.023         | 69.6 ± 2.6      |
| EGCG                | 316.7 ± 5.7   | 1.61 ± 0.05   | 0.51 ± 0.01     | 0.717 ± 0.023         | 0.527 ± 0.035         | 73.7 ± 4.8      |
| EGCG + NAL          | 315.6 ± 4.7   | 1.60 ± 0.03   | 0.51 ± 0.01     | 0.698 ± 0.018         | 0.461 ± 0.037         | 65.7 ± 4.4      |
| EGCG + SPT          | 311.5 ± 5.7   | 1.61 ± 0.05   | 0.52 ± 0.02     | 0.692 ± 0.020         | 0.465 ± 0.032         | 67.3 ± 4.1      |
| EGCG + DPCPX        | 318.8 ± 5.6   | 1.61 ± 0.04   | 0.51 ± 0.01     | 0.723 ± 0.008         | 0.468 ± 0.020         | 64.8 ± 2.8      |
| EGCG + ZM241385     | 316.7 ± 5.7   | 1.60 ± 0.07   | 0.51 ± 0.03     | 0.738 ± 0.023         | 0.487 ± 0.031         | 66.3 ± 4.6      |
| EGCG + MRS1706      | 313.3 ± 4.2   | 1.59 ± 0.05   | 0.51 ± 0.01     | 0.740 ± 0.032         | 0.468 ± 0.018         | 63.5 ± 1.7      |
| EGCG + MRS1334      | 314.9 ± 5.3   | 1.59 ± 0.03   | 0.51 ± 0.01     | 0.693 ± 0.031         | 0.467 ± 0.037         | 66.9 ± 2.4      |

Values are means ± SEM. CON: untreated control heart, EGCG: polyphenol (-)-epigallocatechin gallate, NAL: nonspecific opioid receptor antagonist naloxone, SPT: nonspecific adenosine receptor (ADR) antagonist 8-(p-sulfophenyl)theophylline hydrate, DPCPX: A₁ ADR antagonist, ZM241385: A₂a ADR antagonist, MRS1706: A₂b ADR antagonist, MRS1334: A₃ ADR antagonist, BW: body weight, HW: heart weight, LV: left ventricle, AR: area at risk. There were no significant differences among groups.
fore, we report the data for 90 successfully completed infarct experiments (each group n = 9).

There were no significant group differences in body weight, heart weight, heart to body weight ratio, LV volume, AR volume and AR to LV ratio (Table 1). Ten μM of EGCG targeting reperfusion significantly reduced infarct size over risk area from 33.5 ± 4.1 % to 14.4 ± 1.1% (P < 0.001 vs. CON) (Fig. 3). Ten μM of the nonspecific OPR antagonist naloxone (14.3 ± 1.3%, P < 0.001 vs. CON) could not block the infarct-limitation effect by EGCG. However, 1 μM of the nonspecific ADR antagonist 8-SPT (27.1 ± 1.9%, P > 0.05 vs. CON) blocked the anti-infarct effect by EGCG. Naloxone and 8-SPT itself did not alter infarct size (30.9 ± 4.5% for NAL and 29.6 ± 3.2% for 8-SPT, P > 0.05 vs. CON).

All four ADR antagonists had a tendency to attenuate the infarct-sparing effect by EGCG. The infarct reducing effect of EGCG was significantly reversed by 200 nM of the A₁ ADR antagonist DPCPX (25.9 ± 1.9%, P < 0.01) and 15 nM of the A₂B ADR antagonist MRS1706 (29.3 ± 1.7%, P < 0.01) but not by 10 μM of the A₃ ADR antagonist ZM241385 (23.9 ± 1.9%) and 100 nM of the A₁ ADR antagonist MRS1334 (24.1 ± 1.8%) (Fig. 4).

Discussion

In the present study, EGCG targeting reperfusion effectively reduced infarct size after myocardial ischemia and reperfusion. Interestingly, the infarct reducing effect by EGCG was totally

![Fig. 3](image-url)  
*Fig. 3. (A) Representative sequential left ventricle (LV) slices from each group showing area of necrosis (pale area) with TTC staining. (B) % of infarct area (AN) over area at risk (AR). All data are expressed as means ± SEM. CON: untreated control hearts, EGCG: polyphenol (-)-epigallocatechin gallate, NAL: nonspecific opioid receptor antagonist naloxone, SPT: nonspecific adenosine receptor antagonist 8-(p-sulfophenyl)theophylline hydrate. *P < 0.05 vs. CON, †P < 0.05 vs. EGCG.

![Fig. 4](image-url)  
*Fig. 4. (A) Representative sequential left ventricle (LV) slices from each group showing area of necrosis (pale area) with TTC staining after adenosine receptor (ADR) antagonist pretreatment in EGCG treat hearts. (B) % of infarct area (AN) over area at risk (AR). All data are expressed as means ± SEM. CON: untreated control hearts, EGCG: polyphenol (-)-epigallocatechin gallate, DPCPX: A₁ ADR antagonist, ZM241385: A₂a ADR antagonist, MRS1706: A₂b ADR antagonist, MRS1334: A₃ ADR antagonist. *P < 0.05 vs. CON, †P < 0.05 vs. EGCG.
EGCG and adenosine receptor

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