Mitochondrial DNA-induced inflammatory damage contributes to myocardial ischemia reperfusion injury in rats: Cardioprotective role of epigallocatechin

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Abstract. Inflammation serves an important role in the pathogenesis of myocardial ischemia/reperfusion (I/R) injury. Fragments of endogenous damaged-associated molecular patterns, recently identified as mitochondrial DNA (mtDNA), have been proven to be a potent pro-inflammatory mediator. Epigallocatechin-3-gallate (EGCG) is able to regulate the expression levels of a series of inflammatory cytokines. However, the involvement of endogenous mtDNA in EGCG-regulated inflammatory activities in the context of myocardial I/R injury remains to be elucidated. The present study was designed to investigate the role of mtDNA in EGCG-mediated myocardial protection in a rat I/R model. Significant positive correlations between elevated plasma mtDNA copy numbers and the expression levels of tumor necrosis factor (TNF) and interleukins (IL)-6 and -8 were observed in the myocardial tissue following an I/R injury ($P<0.05$). However, EGCG administered prior to reperfusion was able to effectively downregulate the expression levels of plasma mtDNA, TNF and IL-6 and -8 in the myocardial tissue following an I/R injury ($P<0.05$). Limited infarct size, reduced severity of myocardial injury and decreased incidence of ventricular arrhythmia were observed in the EGCG-treated group. However, the beneficial effects of EGCG in preventing myocardial I/R injury may be eliminated by a specific phosphoinositide-3-kinase (PI3K) inhibitor. These results suggested that EGCG-mediated cardioprotective effects may be achieved by inhibiting the release of mtDNA from damaged mitochondria and that this protection was at least in part dependent on the PI3K/RAC-α serine/threonine-protein kinase associated signaling pathway.

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Introduction

With the increasing threat of acute myocardial infarction worldwide, the past decades have witnessed an advancement in interventional technologies and thrombolytic treatments (1). The goal of these therapeutic interventions is a timely restoration of the blood supply to the ischemic tissue, the salvaging of viable myocardium and limiting the size of myocardial infarction. Typically, the infarct size is associated with the incidence of short- and long-term adverse events in patients with acute myocardial infarction (1-3). However, the reperfusion itself paradoxically causes further cardiomyocyte death and contractile dysfunction, and increases infarct size, a phenomenon known as ischemia/reperfusion (I/R) injury (3,4). Although the underlying mechanisms of myocardial I/R injury remain to be fully elucidated, the inflammatory activities triggered by an acute ischemic injury are hypothesized to be a significant contributor (3-5).

Mitochondrial DNA (mtDNA) is a double-stranded and naked circular DNA and resembles bacterial DNA in containing non-methylated CpG motifs (6). Accumulating evidence has indicated that mtDNA, when released from damaged mitochondria into circulation, is able to act as damage-associated molecular patterns (DAMPs) with inflammation in several pathological conditions (6-8). Previously, phosphoinositide-3-kinases (PI3Ks) and their downstream target, RAC-α serine/threonine-protein kinase (Akt), have been regarded as a negative feedback signaling pathway in the progression of inflammatory responses (9,10). Activation of the PI3K/Akt-dependent pathway has been demonstrated to protect the heart against I/R injury by downregulating the expression levels of a series of pro-inflammatory cytokines (10-12). However, in the context of myocardial I/R injury, the role of the mtDNA-induced inflammatory response and its association with PI3Ks/Akt signaling remains to be elucidated.

Epigallocatechin-3-gallate (EGCG), the most abundant catechin in green tea, may prevent visceral organ I/R injury partially

due to its anti-inflammatory properties (13,14). However, more recent findings have also suggested other beneficial effects of EGCG attributing to its anti-oxidative action for preventing mitochondrial damage (15). Therefore, the present study was designed to investigate whether EGCG administered prior to reperfusion protects the heart against I/R injury, and to determine its potential mechanisms involving the role of mtDNA and PI3Ks/Akt signaling pathway in a rat model.

Materials and methods

Animals. The present study was conducted with the approval of the Institutional Animal Care and Use Committee at West China Hospital of Sichuan University. A total of fifty 10-week-old male Wistar rats weighing 220-250 g were obtained from Hua Fu Kang Experimental Animal Center (Beijing, China). The rats were housed and acclimated in a specific pathogen-free facility with a 12 h light:dark cycle and fed with chow food and water *ad libitum*.

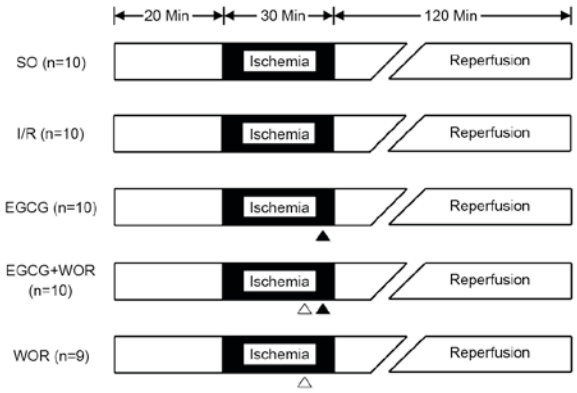

Figure 1. Experimental protocol. Black and white arrows indicate administration of EGCG and WOR, respectively. SO, sham-operated control group; I/R, ischemia and reperfusion group; EGCG, epigallocatechin-3-gallate/epigallocatechin-3-gallate group; EGCG+WOR, EGCG plus wortmannin group; WOR, wortmannin-only group.

Myocardial ischemia and reperfusion preparation. As previously described (11), the rats were anesthetized with pentobarbital sodium (100 mg/kg, intraperitoneally). Rats were subsequently placed in a supine position and secured on an electric heating pad to maintain constant body heat at 37˚C. Catheters were cannulated into the carotid artery and jugular vein for arterial blood pressure monitoring and drug administration. The electrocardiogram was monitored with subcutaneous stainless steel electrodes in the chest. A left thoracotomy through a left parasternal incision was performed to expose the anterior wall of the left ventricle. Following a stabilization period of 20 min, myocardial ischemia was induced by 4-0 silk suture slipknot at the level of the proximal left anterior descending coronary artery for 30 min. Reperfusion was started by releasing the slipknot and the heart was harvested following a 2 h reperfusion.

Experimental protocol. The Wistar rats were randomly allocated into five groups (n=10 in each group; Fig. 1): i) Sham-operated control (SO) group (rats were subjected to surgical manipulation without any induction of I/R injury); ii) I/R group (rats were subjected to the coronary artery occlusion for 30 min followed by 2 h reperfusion); iii) EGCG group (EGCG 10 mg/kg, administered intravenously at 5 min prior to the onset of reperfusion); iv) EGCG + wortmannin (WOR) group (WOR, a PI3K inhibitor, 0.6 mg/kg + EGCG 10 mg/kg administered intravenously at 10 min prior to the reperfusion); and v) WOR group (WOR alone intravenously administered at 10 min prior to the reperfusion). The dosage of EGCG and WOR was determined according to previous studies (11-13).

Measurement of myocardial injury and infarct size. At the end of reperfusion, blood samples were collected and centrifuged at 500 x g for 15 min at 4˚C to assess the serum levels of lactate dehydrogenase (LDH) and creatine kinase (CK) by using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The hearts were subsequently harvested and the left anterior descending artery was occluded again. Diluted fluorescent polymer microspheres (Duke Scientific Corporation, Palo Alto, CA, USA) were infused into the aorta to demarcate the infarct area. Subsequent to being frozen at -20˚C, the hearts were cut into 2 mm transverse sections and incubated in 1% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37˚C for 20 min. The infarct regions and the area at risk were quantified using Image-Pro Plus (version 3.0; Media Cybernetics, Silver Spring, MD, USA). The infarct regions and area at risk were converted into volumes by multiplying them by section thickness.

Western blot analysis. Western blot analysis of the protein levels for phosphorylated (p)-p85, total (t)-Akt and p-Akt in myocardial tissues was carried out as previously reported (14). Antibodies against p-p85 (1:1,000; catalog no. ab182651; Abcam, Cambridge, MA, USA), p-Akt (1:500; catalog no. 4051; Cell Signaling Technology, Inc., Danvers, MA, USA) and t-Akt (1:1,000; catalog no. 2920; Cell Signaling Technology, Inc.) were used to probe the membranes, followed by incubation with a horseradish peroxidase-conjugated secondary antibody (1:500; catalog no. 111-035-003; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). β-actin (1:300; catalog no. A0A068F1Y2; Abmart (Shanghai) Co., Ltd., Shanghai, China,) was used for normalization. The reactive bands were visualized using the ECL-Plus reagent (Amersham Biosciences Corp., Piscataway, NJ, USA). The density of each reactive band was quantified using the Labworks image acquisition platform and its related analytic software (GDS8000; UVP, Inc., Upland, CA, USA).

DNA isolation and quantitative polymerase chain reaction (qPCR) of mtDNA. As previously reported (16), the whole plasma DNA was isolated from plasma using the DNeasy Blood and Tissue kit (69504; Qiagen Inc., Valencia, CA, USA). A total of 50 μl plasma samples were added to 50 μl PBS and centrifuged at 700 x g at 4˚C for 5 min to obtain the supernatant. All procedures were performed according to the manufacturer's protocols. Plasma mtDNA levels were measured by SYBR-Green dye-based qPCR assay (45 cycles of 95˚C for 15 sec and 60˚C for 1 min) using a PRISM 7300 sequence detection system. The primer sequences were rat

Table I. Baseline and intraoperative cardiodynamic data.

Variable	SO (n=10)	I/R (n=10)	EGCG (n=10)	EGCG +WOR (n=10)	WOR (n=9)
SAP, mmHg					
Baseline	109±7	106±9	103±4	106±5	104±3
I-30	110±8	105±4	101±3	105±3	98±3
R-60	104±2	112±5	110±6	106±4	105±5
R-120	105±3	103±3	105±4	105±4	104±3
MAP, mmHg					
Baseline	99±6	95±6	94±4	91±6	95±5
I-30	98±7	92±5	88±5	92±4	89±4
R-60	96±5	94±4	94±4	91±6	92±7
R-120	97±3	94±6	92±6	94±7	91±6
DAP, mmHg					
Baseline	88±5	83±2	85±3	75±3	85±2
I-30	85±4	79±3	74±3	78±3	79±4
R-60	87±4	76±4	77±4	76±3	78±3
R-120	88±3	85±6	79±3	82±4	77±6
HR, beats/min					
Baseline	357±19	346±15	363±19	348±17	353±12
I-30	349±17	354±14	354±18	347±12	347±14
R-60	362±14	362±16	361±15	354±15	349±11
R-120	354±16	358±15	359±17	351±11	362±10

Data are presented as the mean ± standard deviation. SO, sham-operated control group; I/R, ischemia and reperfusion group; EGCG, epigallocatechin-3-gallate group; EGCG+WOR, EGCG plus wortmannin group; WOR, wortmannin-only group; SAP, systolic arterial pressure; MAP, mean arterial tissue; DAP, diastolic arterial pressure; HR, heart rate; I-30, 30 min following the initiation of ischemia; R-60/120, 60/120 min following the initiation of reperfusion.

NADH dehydrogenase 1 gene (mtDNA): Forward CGCCTG ACCAATAGCCATAA and reverse ATTCGACGTTAAAGC CTGAGA. All samples were measured with standards at the same time and standard mtDNA was diluted in 10-fold serial dilutions. All measurements were conducted three times for quality control. Concentration of plasma mtDNA was converted to copy number via a DNA copy number calculator (cels.uri.edu/gsc/cndna.html; University of Rhode Island Genomics and Sequencing Center). Plasma mtDNA levels were shown in copies per μl plasma according to the following formula: $c=QxV_{DNA}/V_{PCR}x1/V_{ext}$, where c is the concentration of plasma mtDNA (copies/μl), Q is the quantity of DNA measured by RT-PCR, V_{DNA} is the total volume of plasma DNA solution obtained from the extraction (200 μl), V_{PCR} is the volume of plasma DNA solution for RT-PCR (1 μl) and V_{ext} is the volume of plasma used for the extraction (50 μl).

Assessment of inflammatory cytokines. Expression levels of TNF-α and IL-6 and -8 in myocardial tissue supernatants were quantified by using specific ELISA kits (BioSource International; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocols. Additionally, total RNA was extracted from myocardial tissues with Trizol, according to the manufacturer's protocol (Takara Bio, Inc., Otsu, Japan). The mRNA from each tissue sample was reverse transcribed with PrimeScript RT Master Mix (Takara Bio, Inc.). qPCR amplifications (45 cycles of 95˚C for 15 sec and 55˚C for 1 min) were carried out using the ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primers used were as follows: TNF-α forward 5'-CTGAACTTCGGGGTG ATCGG-3' and reverse 5'-GGCTTGTCACT CGAATTTTG AGA-3'; IL-6 forward 5'-CTTCCAGCCAGTTGCCTTCT-3' and reverse 5'-GAGAGCATTGGAAGTTGGGG-3'; IL-8 forward 5'-AGCTTCCTTGTGCAAGTGTCT-3' and reverse 5'-GACAGCCCAGGTCAAAGGTT-3'; and β-actin forward 5'-AGAGGGAAATCGTGCGTGAC-3' and reverse 5'-CAA TAGTGATGACCTGGCCGT-3'. The amount of mRNA for each gene was normalized by β-actin and the relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (17).

Statistical analysis. Results are expressed as the mean ± standard deviation. Differences in hemodynamic parameters and infarct measurements between groups were determined by one-way analysis of variance with Tukey's honest significant difference post hoc test. χ^2 analysis was used to test the differences in the incidence of ventricular arrhythmias. The Kruskal-Wallis test was used to compare the means for all nonparametric scores between groups. The Pearson correlation coefficient test was performed. All analyses were performed using SPSS (version 19.0; IBM SPSS, Armonk, NY, USA) and

Table II. Body weight, heart weight and morphometric data.

Variable	SO (n=10)	I/R (n=10)	EGCG (n=10)	EGCG + WOR (n=10)	WOR (n=9)
Body weight, g	236±13	233±12	227±16	241±13	239±15
Heart weight, g	1.13±0.04	1.15±0.03	1.20±0.04	1.16±0.05	1.14±0.04
LV volume, cm^3	0.47±0.04	0.48±0.04	0.49±0.04	0.50±0.04	0.46±0.05
AR volume, cm^3	0.22±0.01	0.22±0.01	0.22±0.01	0.24±0.02	0.24±0.01
AR/LV, %	48.4±5.5	45.3±5.6	46.0±4.7	49.4±3.2	49.3±2.0
AI volume, cm^3	0	0.11±0.01	0.05±0.01[a]	0.10±0.01[b]	0.10±0.01[b]
Infarct size, %	0	50.0±3.2	22.5±4.2[a]	40.2±4.4[b]	43.2±6.0[b]

Data are presented as the mean ± standard deviation. [a]P<0.05 vs. I/R, [b]P<0.05 vs. EGCG. AI, areas of infarction; AR, areas at risk; LV, left ventricle; SO, sham-operated control group; I/R, ischemia and reperfusion group; EGCG, epigallocatechin-3-gallate group; EGCG+WOR, EGCG plus wortmannin group; WOR, wortmannin-only group.

Table III. Incidence of ventricular arrhythmia.

Variable	SO (n=10)	I/R (n=10)	EGCG (n=10)	EGCG + WOR (n=10)	WOR (n=9)
PVCs					
Episodes, n	26.4±17.5	223.8±50.4	111.2±32.2	199.8±49.1	219.8±43.0
VT					
Episodes, n	0	27.2±7.5	14.8±3.6	21.8±5.2	28.0±7.8
VF					
Episodes, n	0	5.6±2.7	1.8±0.8	4.6±1.1	6.0±2.1
Total duration, sec	0	40.2±18.8	6.2±3.3	38.0±16.2	39.2±15.1
VAS	1.4±0.3	8.3±1.0[a]	5.1±0.8[a,b]	8.0±0.6[a,c]	8.4±0.8[a,c]

Data are presented as the mean ± standard deviation. [a]P<0.05 vs. SO, [b]P<0.05 vs. I/R and [c]P<0.05 vs. EGCG. PVC, pre-ventricular contraction; VF, ventricular fibrillation; VT, ventricular tachycardia; VAS, ventricular arrhythmia scores=($\log_{10}$ PVCs) + ($\log_{10}$ episodes VT) + 2[($\log_{10}$ episodes of VF) + ($\log_{10}$ total duration of VF)]; SO, sham-operated control group; I/R, ischemia and reperfusion group; EGCG, epigallocatechin-3-gallate group; EGCG+WOR, EGCG plus wortmannin group; WOR, wortmannin-only group.

P<0.05 was considered to indicate a statistically significant difference.

Results

Heart extraction. A total of 49 rat hearts were successfully harvested for the experiment. One rat in the WOR group was excluded due to an irreversible ventricular fibrillation during the stabilization period. No significant difference in body or heart weight, areas at risk or hemodynamic parameters between groups was observed (Tables I and II).

Severity of I/R-induced ventricular arrhythmia. The episodes and cumulative duration of the premature ventricular contraction, ventricular tachycardia and ventricular fibrillation were recorded. The arrhythmia scoring system suggested by Miller *et al* (18) was used to assess the severity of I/R-induced ventricular arrhythmia. As presented in Table III, ventricular arrhythmia scores in the I/R, EGCG+WOR and WOR groups were significantly increased compared with those in the SO and EGCG groups (P<0.05, respectively).

EGCG protects against myocardial I/R injury. The infarct size induced by an I/R injury was significantly reduced by EGCG compared with the I/R group (22.5±4.2 vs. 50.0±3.2%, P<0.05). Administration of PI3K inhibitor (WOR) eliminated the cardioprotective effects of EGCG on the infarct size compared with the EGCG group (40.2±4.4 vs. 22.5±4.2%, P<0.05). However, WOR alone did not affect the infarct size compared with the I/R group (43.2±6.0 vs. 50.0±3.2%, P>0.05), suggesting that the infarct reducing effects of EGCG involves the activation of the PI3K-Akt signaling pathway (Table II). Following a 2 h reperfusion, LDH and CK levels in the I/R group were significantly increased compared with the SO group (P<0.05, respectively). However, a notable decrease of these kinases was observed in the EGCG group (Fig. 2). No significant differences in LDH and CK levels were observed between the WOR and EGCG+WOR group and those in the I/R group.

EGCG prevents mtDNA release caused by I/R injury. mtDNA, as a pro-inflammatory agent, is released following I/R injury to cause inflammatory damage to the heart. In order to study the role of EGCG on mtDNA release, blood samples were

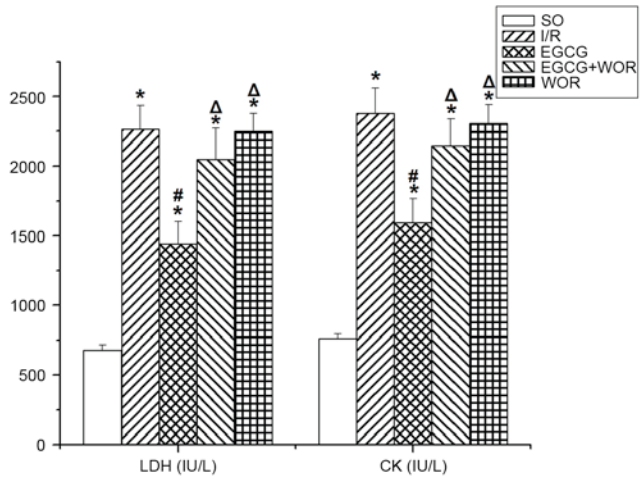

Figure 2. Effects of EGCG on myocardial kinases. Data are presented as the mean ± standard deviation. *P<0.05 vs. SO, #P <0.05 vs. I/R and ΔP<0.05 vs. EGCG. EGCG, epigallocatechin-3-gallate/epigallocatechin-3-gallate group; SO, sham-operated control group; I/R, ischemia and reperfusion group; LDH, lactate dehydrogenase; CK, creatine kinase.

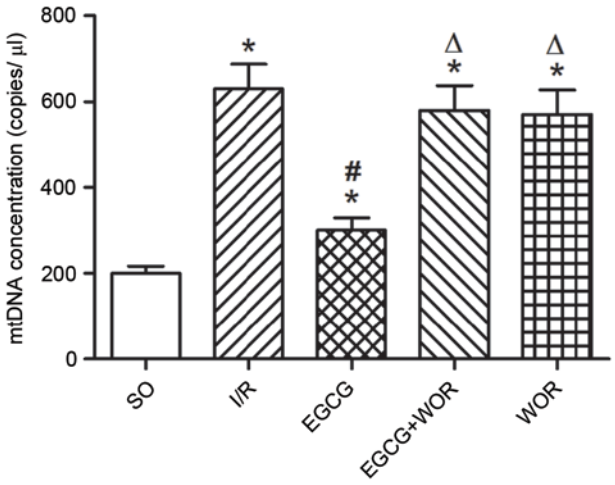

Figure 3. Effect of EGCG on plasma mtDNA level. Plasma mtDNA levels were measured by RT-PCR and presented as copies per microliter. Data are presented as the mean ± standard deviation. *P<0.05 vs. SO, #P<0.05 vs. I/R and ΔP<0.05 vs. EGCG. mtDNA, mitochondrial DNA; RT-PCR, real-time polymerase chain reaction; SO, sham-operated control group; I/R, ischemia and reperfusion group; EGCG, epigallocatechin-3-gallate/epigallocatechin-3-gallate group.

collected from all rats. As demonstrated in Fig. 3, EGCG significantly reduced the plasma levels of mtDNA following I/R injury; 2.13-fold lower compared with the I/R group (P<0.05). Additional treatment with WOR is able to eliminate the mtDNA reducing effects of EGCG, implying that EGCG may exert its inhibitory effect on mtDNA release by activating PI3K-mediated signaling pathways.

EGCG activates the PI3K/Akt signaling pathway. To investigate the role of the PI3K/Akt signaling pathway in the effect of EGCG, the expression of the p-p85 (an activated regulatory subunit of PI3K), p-Akt and t-Akt were examined. As demonstrated in Fig. 4A and B, EGCG significantly increased the expression levels of p-p85 (62.3±4.3% of the I/R group,

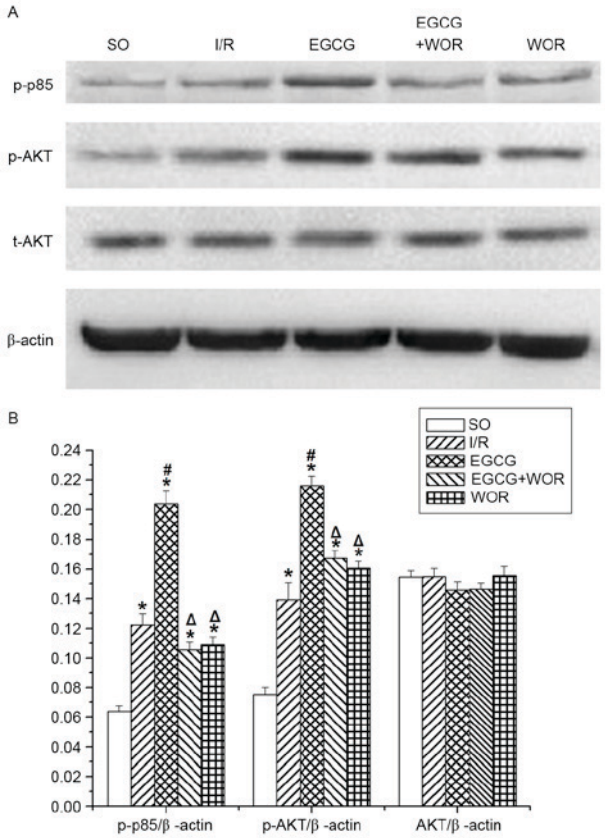

Figure 4. Effects of EGCG on PIK/Akt signaling pathway. (A) Upper panels demonstrate iummunoblots and lower panels densitometry data. (B) Data are presented as the mean ± standard deviation. *P<0.05 vs. SO, #P<0.05 vs. I/R and ΔP<0.05 vs. EGCG. EGCG, epigallocatechin-3-gallate/epigallocatechin-3-gallate group; SO, sham-operated control group; I/R, ischemia and reperfusion group; Akt, RAC-α serine/threonine-protein kinase.

P<0.05) and p-Akt (36.4±6.3% of the I/R group, P<0.05). However, the upregulating effects of EGCG on p-p85 and p-Akt expression were abolished by WOR.

EGCG-activates PI3K/Akt signaling suppresses inflammatory cytokine expression. As demonstrated in Fig. 5A and B, the protein levels of TNF-α, IL-6 and IL-8 in heart tissues were significantly higher in the I/R group than those in the SO group. Consistently, an I/R injury increased the mRNA expression of TNF-α by 3.68-fold, IL-6 by 3.75-fold and IL-8 by 2.89-fold compared with those in the SO group. It is noted that EGCG markedly reduced the protein and mRNA levels of these cytokines. However, the anti-inflammatory effect of EGCG could be inhibited by WOR (P<0.05). Similarly, I/R-stimulated TNF-α, IL-6 and IL-8 overproduction remained unaffected in rat hearts treated with WOR alone, suggesting that the EGCG-activated PI3K/Akt signaling pathway may serve as a negative feedback mechanism in the setting of I/R-triggered inflammatory responses.

Positive correlation between mtDNA level and inflammatory cytokine expression. To further analyze the association between mtDNA and inflammatory cytokines, a bivariate correlation study was performed to examine these parameters in all groups with the exception of the control group. As hypothesized, positive correlations were reached between mtDNA

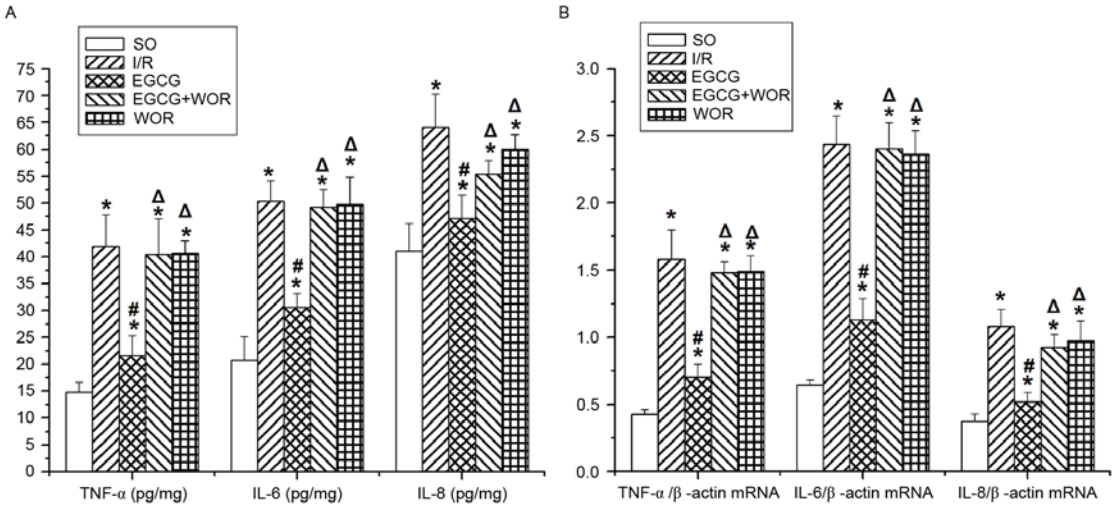

Figure 5. EGCG-induced anti-inflammatory effects. The (A) protein and (B) mRNA levels of TNF-α, IL-6 and IL-8 in myocardial tissue were quantified by using specific ELISA kits and qPCR, respectively. Data are presented as the mean ± standard deviation. *P<0.05 vs. SO, #P<0.05 vs. I/R and ΔP<0.05 vs. EGCG. EGCG, epigallocatechin-3-gallate/epigallocatechin-3-gallate group; TNF, tumor necrosis factor; IL, interleukin; qPCR, quantitative polymerase chain reaction; SO, sham-operated control group; I/R, ischemia and reperfusion group.

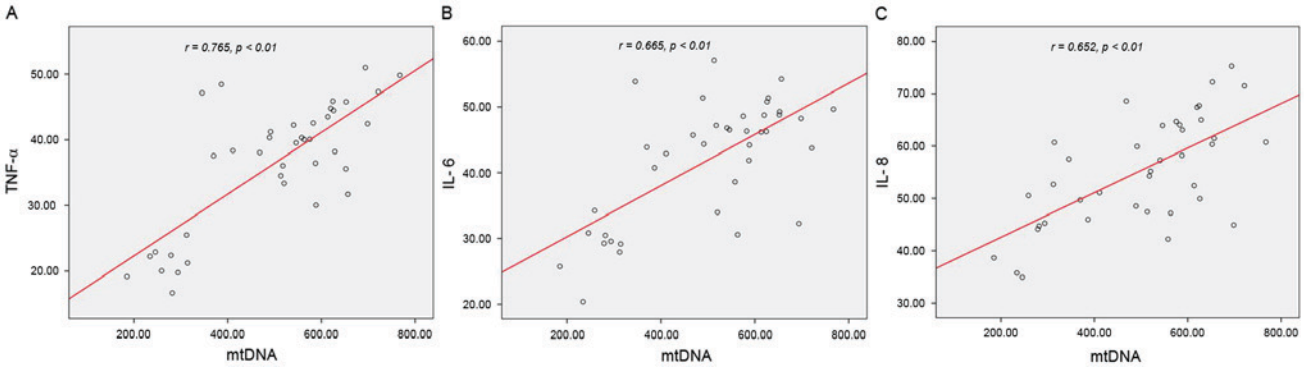

Figure 6. Positive correlations between mtDNA level and inflammatory cytokine expression levels. Bivariate correlation was performed and identified positive correlations between mtDNA level and (A) TNF-α (r=0.765, P<0.01, (B) IL-6 (r=0.665, P<0.01, and (C) IL-8 (r=0.652, P<0.01, in I/R, EGCG, EGCG+WOR, WOR groups. mtDNA, mitochondrial DNA; TNF, tumor necrosis factor; IL, interleukin; I/R, ischemia and reperfusion group; EGCG, epigallocatechin-3-gallate/epigallocatechin-3-gallate group; EGCG+WOR, EGCG plus wortmannin group; WOR, wortmannin-only group.

level and TNF-α (r=0.765, P<0.01), IL-6 (r=0.665, P<0.01) and IL-8 (r=0.652, P<0.01) expression levels (Fig. 6A-C), implying that increasing mtDNA level may contribute to inflammatory cytokine expression levels.

Discussion

The present study revealed the beneficial effects of EGCG postconditioning on preventing myocardial I/R injury in a rat model. EGCG was demonstrated to limit infarct size and reduced the severity of myocardial injury and ventricular arrhythmia effectively. It was also identified that EGCG was able to down regulate plasma mtDNA levels, and the expression levels of TNF-α, IL-6 and IL-8 in myocardial tissue following an I/R injury. In addition, the cardioprotective and anti-inflammatory effects of EGCG were blocked by a specific PI3K inhibitor. Therefore, the results suggested that EGCG-induced anti-inflammatory action may attenuate myocardial I/R injury by reducing mtDNA release under the activation of the PI3K/Akt signaling pathway.

Existing evidence indicated that I/R is able to trigger a vigorous inflammatory response. A series of cytokines generated by infiltrated leukocytes and regional myocytes may further augment the inflammatory cascades and exacerbate myocardial injury (3-5). Consistent with previous studies (8-11), an association was observed between an increased level of classical pro-inflammatory cytokines (TNF-α, IL-6 and IL-8) and a more severe reperfusion-induced myocardial injury in terms of higher ventricular arrhythmia scores, myocardial kinases levels and larger infarct size. The data suggested a contributory role for inflammation in the pathogenesis of myocardial I/R injury. Additionally, the results implied a promising potential in designing therapeutic strategies aimed at suppressing inflammatory responses at the time of reperfusion for an improved cardiovascular outcome.

The cardioprotective effects of green tea have been ascribed consistently to the antioxidant activity of EGCG, its major polyphenolic constituent. Indeed, multiple studies have demonstrated that EGCG could prevent endothelial dysfunction and reduce myocardial I/R injury by inhibiting the release

of reactive oxygen species (12,15,19,20). However, several other studies have also identified that EGCG actually serves as a pro-oxidant in intact cells (21,22), suggesting that the beneficial actions of EGCG may involve mechanisms unassociated with its antioxidant capabilities. The present study identified that EGCG significantly reduced plasma mtDNA levels and the expression levels of TNF-α, IL-6 and IL-8 in the myocardial tissue following an I/R injury. Notably, this reduction was associated with limited infarct size and decreased myocardial kinases levels. These findings indicated the effectiveness of EGCG-induced anti-inflammatory action in protecting hearts against I/R injury.

mtDNA serves as a pro-inflammatory agent, released following cell injury to induce inflammatory responses, featuring high expression levels of TNF-α, IL-6 and IL-8 (16,23,24). It has been reported that abnormally high mtDNA release from oxidative mitochondria is responsible for triggering subsequent inflammatory responses (25). Yao *et al* (26) identified that mtDNA escaped from damaged mitochondria functioned as a type of DAMP to stimulate inflammation through the TL9-RAGE pathway. The data from the present study also demonstrated a significant positive association between the serum level of mtDNA and the expression levels of inflammatory cytokines in all four experiment groups. Given the antioxidant characteristic of EGCG, the results indicated that EGCG could efficiently prevent the release of mtDNA from damaged myocardium, which would further reduce the expression levels of several inflammatory cytokines and to function as a protective agent against I/R injury.

PI3K/Akt is an intracellular signaling pathway and is generally regarded as the primary pro-survival mechanism in the ischemic-reperfused myocardium (6,7,9,10). A series of *in vitro* and *in vivo* studies have proposed that activation of the PI3K/Akt signaling by procedures including ischemic preconditioning or postconditioning, or by administration of pharmacological agents, is critical for protecting the myocardium from lethal I/R-induced cell apoptosis (6,7,12,13,15,19,20,22). Consistent with previous studies, the present study recorded a significant reduction in myocardial kinases and infarct size in rat hearts through the PI3K/Akt-dependent signaling mechanism. The data clearly demonstrate that there is a causal association between EGCG administration and upregulation of p-p85 and p-Akt, suggesting the active involvement of PI3K/Akt signaling pathway in EGCG-induced anti-inflammatory and cardioprotective effects.

There are several limitations in the present study. The reperfusion duration is relatively short compared with several other studies (5,11). As sustained activation of the PI3K/Akt signaling pathway may lead to myocardial fibrosis and hypertrophy (10,22), in turn compromising the blood and oxygen supply of the viable myocytes within the risk zone, the role of EGCG-activated PI3K/Akt signaling in the long-term effects of cardioprotection remains to be elucidated. In addition, preconditioning and postconditioning may provide effective protection against myocardial I/R injury. However, the present study adopted EGCG postconditioning rather than preconditioning. The reason for this was that the postconditioning procedures may be more applicable in clinical practices.

In conclusion, the present study demonstrated that the anti-inflammatory and cardioprotective effects of EGCG postconditioning *in vivo* appear to involve the prevention of mtDNA release. Therefore, EGCG and related compounds may provide a novel therapeutic strategy for attenuating myocardial I/R injury.

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