EGCG Blocked Phenylephrin-Induced Hypertrophy in H9C2 Cardiomyocytes, by Activating AMPK-Dependent Pathway

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INTRODUCTION

Cardiac hypertrophy is an adaptive response to pressure or volume stress, characterized by an increase in the size of individual cardiomyocytes and whole-organ enlargement. Despite this process is initially compensatory, sustained pathologic hypertrophy is deleterious and may lead to heart failure [1-3]. Although there has been remarkable development in diagnosis and management of heart failure, the morbidity associated with this ailment is still a major challenge to the economy and public health [4,5]. Current pharmacological approaches to the treatment of heart failure including calcium channel blockers, beta-blockers and renin-angiotensin-aldosterone system inhibitors. In spite of impressive gains with existing pharmacological treatment, patients still have poor quality of life and suffer serious side effects [6,7]. Therefore, the need to explore novel strategies remains. Recently, Epigallocatechin-3-gallate (EGCG), the major component of polyphenols in green tea, has been reported to be cardioprotective in studies using animal models of cardiovascular disease [8]. Moreover, others and our previous study also reported that EGCG could inhibit cardiac hypertrophy and fibrosis both in vivo and in vitro [9,10]. However, the molecular mechanisms underlying the anti-hypertrophic effect of EGCG remain to be clarified, and little is known about the potential target of EGCG on cardiac hypertrophy.

AMP-activated protein kinase (AMPK) is a serine/threonine kinase that can be activated by cellular stresses and ATP depletion [11,12]. It has been shown that activation of AMPK inhibits the development of cardiac hypertrophy via a number of pathways, such as eukaryotic elongation factor-2 (eEF2), p70S6 kinase (p70S6K), and mammalian target of rapamycin (mTOR) [13,14]. Moreover, in AMPK alpha-2 null mice, cardiac hypertrophy induced by isoproterenol or aortic constriction is significantly larger than in controls and is correlated with p70S6K activation [15, 16]. Therefore, AMPK is an attractive target to treat or prevent cardiac hypertrophy and subsequent heart failure.

ABBREVIATIONS: AMPK, AMP-activated protein kinase; p70S6K, p70S6 kinase; eEF-2, eukaryotic elongation factor-2; EGCG, Epigallocatechin-3-gallate; Nppa, natriuretic peptides type A; BNP, brain natriuretic polypeptide; PE, phenylephrine.
Recently, several lines of evidence demonstrated that EGCG could activate AMPK signaling pathway in cancer cells and in mice [17,18]. However, it is not known whether this regulatory effect is related to a protective role cardiac dysfunction. Therefore, the purpose of this study was to examine the effects of EGCG on phenylephrine (PE)-induced cardiac hypertrophy in H9C2 cardiomyocytes, and we further investigated whether the AMPK-dependent pathway is involved.

Table 1. Primer sequences for qRT-PCR

| Primer | Forward | Reverse |
|--------|---------|---------|
| ANF    | 5’-GAAGCTCAACCCGCTCTCA-3’ | 5’-AGCCCTCAGTTGGCTTTT-3’ |
| BNP    | 5’-TTTGGGCGAGAGATAGACC-3’ | 5’-AGAAGAGCCGAGCAG-3’ |
| GAPDH  | 5’-AGAGCTAGAAAACCGTTGAG-3’ | 5’-CTGGGATGGAATTGTGAG-3’ |

METHODS

Reagents

EGCG (purity >95%) was purchased from Sigma aldrich. Dulbecco’s Modified Eagle Medium (DME) and foetal bovine serum (FBS) were purchased from Gibco (Logan, UT, USA) and Sijiqing Biological Engineering Materials Co Ltd. (Hangzhou, China), respectively. Bovine serum albumin (BSA) was purchased from Sangon (Shanghai, China). Antibodies against AMPK, phospho-AMPK (T172) and phospho-p70S6K (T389) were purchased from Cell Signaling (Cell Signaling Technology); anti-β-actin antibody was purchased from Santa Cruz Biotech (Santa Cruz Biotechnology); anti-phospho-eEF2 and total-eEF2 was purchased from bioworldle (Guangzhou, China). EGCG was dissolved in distilled water at 100 μM, and stored at −20°C until dilution before use.

Cultures of H9C2 cardiomyocytes

H9C2 rat cardiomyocytes were cultured in DMEM supplemented with 10% FBS. Cell culture media were changed every 2 days. Before PE stimulation, H9C2 cells were cul-
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Fig. 2. EGCG treatment increases AMPK phosphorylation. (A and B) H9C2 cardiomyocytes were treated with various concentrations of EGCG for 24 h. The levels of phosphor-/total AMPK were detected. (C and D) Cells were treated with 25 μM EGCG for indicated time points and the protein expression of phosphor-/total AMPK were assessed by western blotting. (E and F) Cells were preincubated with 25 μM EGCG for 1 h followed by stimulation with 100 μM PE for 24 h, and then the Levels of phosphor-/total AMPK were measured by western blotting. *p<0.05 vs. the group without treatment, #p<0.05 vs. the group treated with PE, n=3.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from tissues or cultured cells was extracted using Trizol reagent (Invitrogen). One microgram of total RNA was reverse transcripted using One-step RT Kit (Takara Biotechnology) and the resulting cDNA was used as a PCR template. The mRNA expression levels were determined using SYBR-Green Quantitative PCR Kit (Takara Biotechnology) by Applied Biosystems 7500 Fast Real-Time PCR system (ABI). All PCRs were done in triplicate.

Western blotting analysis

Western blotting analyses were performed as previously described [19]. Briefly, protein was separated by SDS-PAGE gel electrophoresis, and then transferred to PVDF membranes (Millipore). After blocking with 5% nonfat milk, the membranes were incubated with primary antibodies, followed by incubation with appropriate horseradish perox-
Fig. 3. EGCG decreases the p70S6K phosphorylation and increases the eEF2 phosphorylation. Cells were preincubated with 25 μM EGCG for 1 h followed by stimulation with 100 μM PE for 24 h. (A and B) The Levels of phospho/total p70S6K were measured by western blotting. (C and D) The phospho/total eEF2 were detected by western blotting. *p<0.05 vs. the group without treatment, #p<0.05 vs. the group treated with PE, n=3.

**Measurement of cell surface area**

Cardiomyocytes grown in 48-well plates were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, followed by 0.5% Triton-100 treatment for 5 min. After incubated with 0.1% rhodamine-phalloidin for 30 min, the myocytes were washed in PBS for further interaction with DAPI. The images of cardiomyocytes were detected by High Content Screening system (ArrayScanVTI, Thermo Fisher Scientific, Rockford, Illinois, USA), and the cell surface area from randomly selected fields (50 for each group) was determined by the built-in image analysis software.

**Statistical analysis**

Data are presented as mean±SE. Statistical analyses between two groups were performed by unpaired Student’s t-test. Differences among groups were tested by one-way analysis of variance (ANOVA). In all cases, differences were considered statistically significant with p<0.05.

**RESULTS**

**EGCG blocks cardiac hypertrophy in H9c2 cardiomyocytes**

In H9c2 cardiomyocytes, EGCG could markedly suppress PE-induced Nppa and BNP gene expression as revealed by qRT-PCR analysis (Fig. 1A). In addition, PE stimulation (100 μM for 24 h) led to significant increase in cell surface area, which could be attenuated by preincubation with EGCG (25 μM) for 60 min and subsequently treated with 100 μM PE (in the presence of EGCG) for 24 h (Fig. 1B and C). However, EGCG itself had no effect on the Nppa and BNP mRNA levels, or surface area of cardiomyocytes.

**EGCG Treatment increases AMPK phosphorylation and decreases the phosphorylation of p70S6K and eEF2**

To evaluate a possible involvement of AMPK, we assessed the phosphorylation status of this key metabolic regulator. Treatment with EGCG led to a induction of AMPK phosphorylation in a time- and dose-dependent manner (Fig. 2A–D). In order to investigate the molecular signaling mechanisms that may contribute to the EGCG-induced reduction in hypertrophic growth, cell lysates from cardiac myocytes

idase (HRP)-labeled second antibodies. Immunoreactive bands were detected with the Super-Signal West Pico Chemiluminescent Substrate (Pierce), and molecular band intensity was determined by densitometry.
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AMPK participates in the inhibitory effects of EGCG on cardiac hypertrophy in H9C2 cardiomyocytes

To examine the role of AMPK pathway in the inhibitory effects of EGCG on cardiac hypertrophy, the activity of AMPK in H9C2 cardiomyocytes was blocked by a selective inhibitor (Compound C) prior to the treatment with EGCG. Western blotting analysis showed that the phosphorylation status of AMPK was significantly suppressed after treated with compound C as compared with control (Fig. 4A). Moreover, EGCG failed to inhibit PE-induced increase in the phosphorylation level of p70S6K and EGCG also failed to increase the phosphorylation status of eEF2 inhibited by PE, which suggested involvement of AMPK (Fig. 4E). Then the cell surface area and mRNA levels of Nppa and BNP were subjected to immunoblot analysis using anti-phospho-AMPK antibodies. As shown in Fig. 2E and F, stimulation with 100 μM PE for 24 h resulted in downregulation of AMPK phosphorylation, which could be reversed by pretreatment with 25 μM EGCG for 60 min. Moreover, although PE increased the phosphorylation status of p70S6K compared with control, EGCG was able to decrease p70S6K phosphorylation to basal levels (Fig. 3A and B). Furthermore, immunoblot analysis of eEF2 using anti-phospho-eEF2 antibody as a marker of its activity (where phosphorylation indicates inactivation) demonstrated that EGCG increased eEF2 phosphorylation in cells in the absence or presence of PE (Fig. 3C and D).
were measured respectively. As shown in Fig. 4B–D, when AMPK was inhibited by compound C, EGCG failed to inhibit PE-induced increase in cell surface area as well as expression of hypertrophic biomarkers, Nppa and BNP, which suggested involvement of AMPK in the protective effects of EGCG against cardiac hypertrophy.

**Role of reactive nitrogen species in EGCG-induced AMPK activation**

To investigate whether RNS is involved in the EGCG-mediated AMPK regulation in this study, the H9C2 cardiomyocytes were pretreated with L-NAME (a nitric oxide synthase inhibitor) for 1 h followed by EGCG stimulation for 24 h. The protein expression of p-AMPK and AMPK were detected by western blotting. As shown in Fig. 4F, pretreatment with an L-NAME prevented the EGCG enhanced phosphorylation of AMPK. In addition, we showed that treatment of H9C2 cardiomyocytes with catalase did not alter AMPK phosphorylation, indicating that ROS are not involved in EGCG-induced AMPK activation in H9C2 cardiomyocytes.

**DISCUSSION**

Whereas previous studies demonstrated that EGCG is effective in preventing cardiac hypertrophy both in cultured cells and in animal models, the mechanisms involved in the anti-hypertrophic effect have not been clearly defined. Our data showed that EGCG is able to activate AMPK and diminish the activity of both p70S6K and eEF2 in H9C2 cardiomyocytes. Moreover, we further showed that the blockade of AMPK activation prevents EGCG suppression of cardiac hypertrophy, suggesting that EGCG exerts its anti-hypertrophic effects via AMPK activation. These findings shed new light on the mechanism underlying anti-hypertrophic effect of EGCG and support the concept that EGCG could be an effective preventive and therapeutic candidate against cardiac hypertrophy.

EGCG and green tea have been shown to exert remarkable protective effects against the cardiovascular system, including an anti-inflammatory effect [20], anti-oxidative [21] and reducing evolving atherosclerosis [22]. In 2003, Priyadarshi et al. first demonstrated that green tea extract essentially blocked the development of cardiac hypertrophy in the 5/6 nephrectomy rat model [23]. However, the green
Dysfunction of cardiac epi- myocytes by EGCG, and is required for EGCG sup-

by which hypertrophic growth can be controlled. In this

There is increasing evidence for the involvement of AMPK 

sponse to the depletion of ATP by cellular stresses [24,25].

regulator of energy homeostasis, which stimulates ATP-generat-

ting pathways and inhibits ATP-consuming pathways in re-

lator of energy homeostasis, which stimulates ATP-generat-

trophic effects remains unclear. AMPK is a critical regu-

pathway (Fig. 4G). This study is relevant to the under-

provide some useful information for elucidating the signaling 

pathways leading to cardiac hypertrophy mediated by PE. 

However, it is currently still unclear how EGCG exactly 

activates AMPK. Future investigation on this matter may 

provide new therapeutic approaches for the management 

of cardiac hypertrophy.

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