Epigallocatechin Gallate Attenuated the Activation of Rat Cardiac Fibroblasts Induced by Angiotensin II via Regulating β-Arrestin1

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Key Words
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Abstract
Background/Aims: Angiotensin II (AngII) activated cardiac fibroblasts (CFs) predominantly through AngII subtype 1a receptor (AT1aR). This study was carried out to explore the potential inhibitory effects and mechanisms of epigallocatechin gallate (EGCG) on AngII induced rat CFs. Methods: Viability, proliferation and collagen production of CFs were measured by MTT assay, [3H]-thymidine and [3H]-proline incorporation respectively. β-arrestin1 (βarr1), AT1aR and AT1bR mRNA levels were determined by quantitative PCR. AT1R, Gq, βarr 1/2, phosphorylated kinase C (p-PKC)-delta expressions were detected by western blotting. We blocked βarr1 expression using βarr1 small interfering RNA (siRNA). Results: EGCG inhibited the activation of CFs induced by AngII. βarr1 mRNA level revealed a positive correlation with the viability of CFs. SiRNA targeting βarr1 blocked the activation of CFs. In vitro, AngII increased βarr1 mRNA, total and membrane βarr1 protein expressions, but reduced AT1aR mRNA, global and membrane AT1R, total Gq and cytoplasmic p-PKC-delta levels. Administration of EGCG restored the above abnormalities, whereas Gq levels were not affected. Conclusion: Our findings showed that βarr1 is essential for AngII-mediated activation of CFs. EGCG attenuated CFs activation induced by AngII via regulating βarr1 and thus, modulating AT1aR mediated signaling.
Introduction

Myocardial remodeling is a continuum of changes in the structure and function of the myocardium that commonly occur as a result of a pathological process [1]. During myocardial remodeling, cardiac fibroblasts (CFs) play a central role in the maintenance of extracellular matrix (ECM) and undergo hyperplasia in response to some humoral factors such as angiotensin II (AngII) and endothelin (ET) [2]. The effects of AngII on CF were found to be exclusively mediated via angiotensin type 1a receptor (AT1aR), which is a G protein coupled receptor (GPCR) [3].

GPCRs are extremely important drug targets; intracellular scaffolding and adaptor proteins such as β-arrestins (βarrs) regulate major aspects of their pharmacology. Exciting new data have revealed that βarrs play potential roles as key signaling molecules in the treatment of heart failure. Beta-arrestin1 and βarr2 mRNA levels were induced in the hearts of rats with congestive heart failure [4]. Based on considerable evidence accumulated during the last few years, much attention is focused on the use of naturally occurring botanicals for the prevention of heart diseases. Epigallocatechin gallate (EGCG) is the most abundant catechin in green tea and is considered as a potent antioxidant that may have therapeutic applications in the treatment of cardiac disorders [5]. The existing results mainly focused on the antioxidation effects of EGCG, less attention had been paid on its role in regulating AT1R mediated signaling [6]. Accordingly, in the studies described here, we investigated the role of βarr1 in CFs activation and the effect of EGCG on it. The results will not only demonstrate the importance of βarr-dependent regulation in AT1R-mediated responses of CFs, but also shed new light on the molecular pharmacological potential of EGCG in treating myocardial remodeling.

Materials and Methods

Materials

EGCG (purity ≥ 95%), angiotensin II, valsartan (Val) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H tetrazolium bromide (MTT) were from Sigma Chemical Co. (St. Louis, MO). [3H] thymidine and [3H]-proline were purchased from Academia Sinica, China. The antibodies against βarr1/2, AT1R, Gq, and phosphorylated kinase C (p-PKC)-delta were the products of Cell Signaling Technology or Santa Cruz Biotechnology. Pan cadherin antibody was purchased from Abcam (UK). Chemically synthesized, double-stranded βarr1 siRNA and SYBR Green were the products of Qiagen China Co., Ltd. First Strand cDNA Synthesis Kit was purchased from Fermentas International Inc. (Burlington, Ontario).

Primary CFs isolation and culture

All studies were approved by the University Ethics Committee for Animal Experiments and conformed to the Guide for the Care and Use of Laboratory Animals published by National Institutes of Health. Primary CFs were isolated from 2-day-old neonatal Sprague-Dawley rat hearts (Animal Department of Anhui Medical University, China, certificate No. 006). All CFs used in our experiments were at passage 2-4 [7].

CFs viability detection

The viability of CFs was evaluated by MTT assay. CFs were seeded into 96-well plates and grown to confluence, synchronized and then cultured with EGCG (1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, 1×10^{-5} mol/L) or valsartan (1×10^{-6} mol/L) in the presence of AngII (1×10^{-7} mol/L) for 24 hours. MTT was added into each well and the plates were incubated for another 6 hours. The formazan crystals were dissolved by 120µl dimethylsulfoxide. The absorbance (A) was measured at 490nm using Bio-Rad EIA Analyzer (Bio-Rad).

CFs proliferation and collagen synthesis measurement

The CFs proliferation and collagen synthesis were measured by [3H]-thymidine and [3H]-proline incorporation assay respectively as described previously. CFs were cultured with different concentrations of EGCG or Val in the presence of AngII for 24 hours in media containing 1 µCi/ml [3H]-thymidine or
2 µCi/ml [3H]-proline. Then culture supernatant was discarded, cell layer was washed with PBS, lysed in lysis buffer (0.1 M NaOH containing 0.1% SDS). Radioactivity was determined using a liquid scintillation counter (LS6500, Beckman, US) [8].

The expressions of βarr1, AT1aR and AT1bR mRNA determination
The total RNA was prepared using Trizol reagent (Invitrogen, Carlsbad, CA). First strand cDNA was synthesized from total RNA (1 µg) by reverse transcription (Fermentas). The primers used for Q-PCR were: AT1aR (forward: 5'-GGA TGG TTC TCA GAG AGA GTA CAT-3', reverse: 5'-CCT GCC CTC TTG TAC CTG TTG-3'), AT1bR (forward: 5'-CTG CCC CAA GGC TGG CAG GC-3', reverse: 5'-GCC ACC TAC TGC TGG CCG TT-3'), βarr1 (forward: 5'-AGG GGA CAT GAG TGT TCA AG-3', reverse: 5'-AGG TGG AAT GGG AGT GAG AT-3'), GAPDH (forward: 5'-TCA AGA AGG TGG TGA AGC AG-3', reverse: 5'-AGG TGG AAG AAT GGG AGT T G-3') served as housekeeping gene [9, 10].

Western blot analysis
CFs were lysed in lysis buffer at 4°C. The homogenate was centrifuged for 4 min at 3000×g and the resulting supernatants were used to detect total protein expression. Part of the supernatant was subjected to centrifugation at 37,000 g for 25 min. The pellet was resuspended in lysis buffer for membrane protein detection. The supernatant was used to determine the expression of cytoplasmic protein. Pan cadherin was applied as a general membrane marker to identify the efficiency of protein extraction. Equal amount of the extracts were loaded to detect the expression of AT1R, Gq, βarr1, βarr2 or p-PKC-delta [11].

Beta-arrestin 1 small interfering RNA (siRNA) transfection
CFs were seeded onto 6-well plates at a density of 2 ×10^4 cells/well and allowed to grow until 80-90% subconfluent, then transfected with 20 µg of siRNA using 20 µl of Lipofectamine 2000. Six hours after transfection, cell layer was washed with PBS and cultured for another 48 hours. CFs were synchronized for at least 48 hours prior to stimulation. The siRNA sequences targeting βarr1 were 5'-AGC CUU CUG UGC UGA GAA C -3', a non-silencing RNA duplex 5'-AAU UCU CCG AAC GUG UCA CGU-3' was used as a control [12].

Statistical Analysis
For all experiments, at least triplicate determinations were made. All data are expressed as mean and standard deviation (SD). Comparisons between two experimental groups were performed using Student's t test, for multiple group comparisons we used ANOVA test. Results were considered statistically significant for P values less than 0.05.

Results
Effects of EGCG on the viability, proliferation and collagen synthesis of AngII-induced CFs
As mentioned above, activation of CFs is an important pathological phenomenon during cardiac hypertrophy and remodeling [1]. New born rat primary CFs were cultured with EGCG (1x 10^-5 mol/L) alone or in presence of AngII, in addition, they were treated with different concentrations of EGCG or Val. Val is an angiotensin II receptor inverse agonist, with particularly high affinity for AT1 receptor. Of note, EGCG alone has no obvious effect on viability, proliferation and collagen production of normal CFs (Fig. 1 A). EGCG at 1×10^-7-1×10^-5 mol/L concentration-dependently inhibited cell viability evoked by AngII, Val displayed a similar effect (Fig. 1 B). As shown in Fig. 1 C and D, in AngII treated CFs, [3H]-thymidine and [3H]-proline incorporation obviously increased. EGCG and valsartan partially, but significantly attenuated the hyperplasia and collagen synthesis of CFs induced by AngII.

The correlation between the inhibitory effect of EGCG on AngII-mediated CFs viability and βarr1 mRNA levels
The expression of βarr1 mRNA was increased in CFs when exposed to AngII, this abnormality was alleviated by the administration of EGCG (1×10^-6, 1×10^-5 mol/L), to varying degrees (Fig. 2A), however, we have not found any changes in βarr2 protein expression in

Statistical Analysis
For all experiments, at least triplicate determinations were made. All data are expressed as mean and standard deviation (SD). Comparisons between two experimental groups were performed using Student's t test, for multiple group comparisons we used ANOVA test. Results were considered statistically significant for P values less than 0.05.
AngII-induced or EGCG treated cells (Fig. 2B and C). Subsequently, the correlation between the inhibitory effect of EGCG on CFs viability (Fig. 1A) and βarr1 mRNA levels (Fig. 2A) was analyzed. Interestingly, the scatter plot of βarr1 mRNA levels and the viability of CFs revealed a significantly positive correlation (Fig. 2D) (R²=0.9304, P=0.0019). This result indicates that the inhibitory effect of EGCG on CFs is related to βarr1 gene expression.

The role of βarr1 in Ang II-induced CFs viability, proliferation and collagen synthesis and the effect of EGCG

To further confirm the role of βarr1 in promoting CFs activation, we knocked down βarr1 gene expression by siRNA and then treated the cells with Ang II (1×10⁻⁷ mol/L) and/or EGCG (1×10⁻⁵ mol/L). Figure 3A and B showed that βarr1 in CFs were reduced by about 55% in response to introducing βarr1 siRNA as compared with the negative control cells. The βarr1 gene disruption blocked the viability, proliferation and collagen production of CFs treated with Ang II. In βarr1 knocked down CFs, EGCG further reduced the viability and proliferation of Ang II treated cells (Fig. 3C-E). These results indicate that βarr1 is essential for AngII-mediated activation of CFs and it substantially contributes to the function of EGCG.

Effects of EGCG on AT1R, βarr1 and Gq expressions

As we known, the effects of Ang II on CFs were found to be exclusively mediated via AT1R and βarr1 is the pivotal negative controller in AT1R signaling, therefore, the effects of EGCG on AT1R signaling were elucidated in the following experiments. As detected, AT1aR mRNA was reduced by AngII stimulation, but was restored to normal levels by the administration of EGCG (1×10⁻⁵ mol/L) (Fig. 4A), whereas expression of AT1bR mRNA was unaltered (Fig. 4B).
Fig. 2. The effects of EGCG on βarr1 and βarr2 expression and the correlation between CFs viability and βarr1 mRNA level. A: The levels of βarr1 mRNA were determined by Q-PCR. B and C: The expression of βarr2 was measured by western blot. D: The scatter plot of βarr1 mRNA levels and the viability of CFs revealed a significantly positive correlation. Data are means ± SD of three experiments. ##P<0.01 vs normal cells; **P<0.01 vs AngII induced CFs.

As shown in Fig. 4C, the purification of membrane protein by the protocol described above was satisfied. In CFs cultured with AngII, total and membrane βarr1 levels were increased (P<0.01, Fig. 4D and E), on the contrary, the expressions of total and membrane AT1R and Gq protein were decreased (Fig. 4F and G). EGCG (1×10^{-6}, 1×10^{-5} mol/L) decreased membrane βarr1 levels, but promoted global and membrane AT1R expressions. EGCG had no obvious effects on Gq expression.

Effects of EGCG on p-PKC-delta expression

The phosphorylation of AT1R by AngII prevents activation of PKC [13]. We then investigated the effects of EGCG on the expression of p-PKC-delta in the membrane and cytosol of CFs. The results showed in Fig. 5 indicated that the p-PKC-delta expression in the cytosol was significantly inhibited by AngII (1×10^{-7} mol/L), as compared with control CFs. Administration of EGCG (1×10^{-6}, 1×10^{-5} mol/L) significantly restored the reduced p-PKC-delta levels. Neither AngII nor EGCG obviously affected the expression of membrane p-PKC-delta.

Discussion

A characteristic feature of fibrotic myocardial remodeling in the injured and failing heart is the abnormal proliferation of CFs, the excessive accumulation of ECM and the persistence of pro-inflammatory cytokines and vasoactive peptides such as Ang II, endothelin and natriuretic peptides [14]. The signal transduction pathways activated by AngII and
responsible for cardiac fibrosis have been deeply explored [15, 16]. In the present study, we found that Ang II directly promoted CFs viability, proliferation and collagen production, which were blocked by EGCG or valsartan. These results indicate that EGCG may target AngII dependent signaling. Therefore, we tried to investigate the potential mechanisms of EGCG in regulating AT1aR signaling.

As we found, the AT1aR plays an important role in myocardial remodeling. It couples with Gq to activate phospholipase C, release calcium, and then activate PKC [17]. The AT1aR is a member of GPCRs superfamily, βarrs specifically bind to phosphorylated active receptors, terminating G protein coupling, internalizing the receptors into clathrin-coated vesicles and establishing a secondary signaling complex independent of G protein signaling [18, 19]. We revealed a significantly positive correlation between the inhibitory effect of EGCG on CFs viability and βarr1 mRNA expression, which indicates that βarr1 is important for the effects of EGCG. The following experiments showed that βarr1 gene silencing in CFs limited the ability of AngII in inducing cell viability, proliferation and collagen synthesis, which further confirmed this interrelation. These findings strongly suggest that βarr1 is essential for AngII-mediated activation of CFs, thus blockade of βarr1 in CFs might serve as a novel therapeutic strategy.

By which mechanisms does βarr1 promote AngII-mediated CFs activation? Upon exposure to AngII, the AT1R uncouples from its G proteins and desensitizes via a βarr1-dependent process. Association with βarr1 is also important for AT1R-dependent ERK1/2 activation [20]. The activation of the ERK1/2 cascade underlies the Ang II-induced proliferation of CFs.
Lymperopoulos reported that increased βarr1 activity resulted in a marked elevation of circulating aldosterone levels in otherwise normal animals, which produces a multitude of effects in vivo, including promotion of postmyocardial infarction adverse cardiac remodeling and heart failure progression [22]. These may represent potential mechanisms for βarr1 in promoting AngII-mediated CFs viability, proliferation, and collagen production. Previous investigations reported that EGCG inhibits the proliferation of CFs both in vivo and in vitro, thereby preventing myocardial fibrosis in cardiac hypertrophy via inducing nitric oxide production [23]. In the present series of studies, we investigated whether the inhibitory effects of EGCG on CFs were associated with modulation of the key AT1R-mediated signal transduction pathways in CFs. The results showed that in Ang II-induced CFs, βarr1 mRNA expression was increased, but AT1aR mRNA level was decreased. We assumed that, as reported by Everett and colleagues, Ang II is a potent negative regulator of the AT1aR gene...
in CFs by two complementary mechanisms involving cAMP and intracellular calcium [24]. In addition, βarr1 substantially contributes to the function of EGCG.

Immunoblotting analysis showed that total and membrane βarr1 expressions in CFs cultured with AngII were increased, however, total and membrane AT1R, global Gq were decreased. These findings suggest the internalization of AT1R is associated with βarr1 translocation. The reduction of membrane AT1R attenuated the activation of Gq and thus, decreased cytoplasmic p-PKC-delta expression. CFs contain alpha-, delta-, epsilon-, betaI-, betaII-, and zeta- PKC isozymes. The observed decrease of activated PKC-delta in AngII stimulated CFs is in agreement with a previous study by Braun et al, who revealed a negative role for PKC-delta in regulating the proliferation of neonatal rat CFs [25].

In summary, our results demonstrate that βarr1 is essential in AngII induced viability, proliferation and collagen synthesis of rat CFs, thus plays an important role in myocardial remodeling. Administration of EGCG may be of value in suppressing the AngII induced activation of rat CFs mainly dependent on blocking AT1R signaling through inhibiting βarr1 expression and its recruitment to cell membrane. Beta-arrestin1 may be a useful therapeutic modality to prevent cardiac remodeling. Nevertheless, how does such a green tea polyphenol contribute to the regulation of βarr1 expression and translocation in rat CFs need to be fully elucidated.

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