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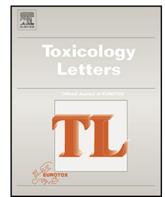

Chlorogenic acid prevents isoproterenol-induced hypertrophy in neonatal rat myocytes

Yanfei Li[a,b], Dan Shen[b], Xiaomei Tang[b], Xin Li[b], Da Wo[b], Hongwei Yan[b], Rui Song[b], Jian Feng[b], Ping Li[a], Jie Zhang[b,*], Jue Li[b,*]

[a] School of Life Sciences and Technology, Tongji University, Shanghai, China
[b] Tongji University School of Medicine, Shanghai, China

HIGHLIGHTS

- Chlorogenic acid (CGA) inhibits isoproterenol (Iso)-induced increase of cell area in neonatal rat myocytes.
- CGA reduces the expression of the hypertrophic markers induced by Iso in cardiomyocytes.
- CGA blocks the translocation of NF-κB to the nucleus.
- CGA suppresses ROS induced by Iso in cardiomyocytes.

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ABSTRACT

Cardiac hypertrophy is an independent risk factor for cardiovascular disease and its subsequent progression to heart failure represents a major cause of morbidity and mortality in the world. CGA is an important component of Chinese herbal medicine, acting as an antioxidant, scavenging free radicals and preventing inflammation. This study found that with the pre-treatment of chlorogenic acid in Iso-induced neonatal rat myocytes, the levels of the hypertrophic markers, ANP, BNP and β-MHC decreased. The nuclear translocation of NF-κB was blocked, whereas NF-κBIA, an inhibitor of NF-κB, was upregulated accordingly. And the level of the intracellular ROS was also reduced. These data reveal that chlorogenic acid may inhibit Iso-induced cardiac hypertrophy by attenuating NF-κB signaling pathway and suppressing ROS.

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1. Introduction

Cardiac hypertrophy leading to heart failure is one of the major causes of morbidity and mortality in the world and is a common pathology in many cardiovascular diseases (Chowdhury et al., 2013; Kanno et al., 2013; Li et al., 2013a,b). Cardiac hypertrophy is characterized by an increase in cell size and is initially an adaptive response to preserve cardiac function (Lu et al., 2013a,b). Constant stimulation with various physiological or pathological factors will ultimately lead to myocardial ischemia, arrhythmias and heart failure (Lu et al., 2013a,b; Savergnini et al., 2013). The levels of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), beta-myosin heavy chain (β-MHC) are biomarkers for cardiac hypertrophy and heart failure, which increase with the progression of cardiac hypertrophy (Iqbal et al., 2013). Isoproterenol (Iso), a type of β-adrenergic receptor agonist, represents the most widely used model which mimics the sustained adrenergic stimulation and represents an important hallmark for the pathogenesis of cardiac hypertrophy (Chowdhury et al., 2013; Molojavyi et al., 2010).

Chlorogenic acid (CGA) is a type of polyphenol, which has anti-inflammatory, anti-oxidative and anticancer properties (Feng et al., 2005; Lambert et al., 2005; Roche et al., 2005). CGA could inhibit inflammatory cell infiltration, notably neutrophil recruitment into lung (Zhang et al., 2010) and inhibit inflammatory cytokines release through suppressing nuclear factor kappa B (NF-κB) activation

Abbreviations: CGA, chlorogenic acid; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; β-MHC, beta-myosin heavy chain; Iso, isoproterenol; NF-κB, nuclear factor kappa B; NF-κBIA, nuclear factor I kappa B alpha; ROS, reactive oxygen species; cTnT, cardiac troponin T.

* Corresponding authors at: School of Life Sciences and Technology, Tongji University, 1239 Siping Road, Shanghai, China. Tel.: +86 21 65985195; fax: +86 21 65985195.
E-mail addresses: jiezhang@tongji.edu.cn (J. Zhang), jueli@tongji.edu.cn, lijue20131001@126.com (J. Li).

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(Shan et al., 2009). In cardiac studies, one study demonstrated that CGA can attenuate chronic ventricular remodeling after myocardial ischemia through suppression of macrophage infiltration (Kanno et al., 2013). Another study showed that chlorogenic acid in American ginseng berry extract up regulated the capacity of cultured cardiomyocytes to metabolize H_2O_2 (Mehendale et al., 2006). The effect of CGA on cardiac hypertrophy has not yet been reported. Based on these findings, we propose CGA can protect Iso-induced cardiac hypertrophy.

The molecular mechanism for the development of cardiac hypertrophy is yet unclear. Some argue that regeneration of capillaries in the heart plays a major role in the development of cardiac hypertrophy (Batra et al., 1991; Kayar and Weiss, 1992; Waller et al., 2008). Some suggest that apoptosis is directly related with cardiac hypertrophy (Sheng et al., 2013; Wencker et al., 2003). Others claim that inflammation caused by pressure overload or oxygen free radicals play a crucial role (Oka et al., 2012). And it has been also reported that the pathogenesis of cardiac hypertrophy was associated with increased oxidative stress (Liu et al., 2010). It has been reported that NF-κB is associated with vascular remodeling, apoptosis, inflammation and oxidative stress (Castier et al., 2009; Kaltschmidt et al., 2000; Lawrence, 2009; Li et al., 2013a,b). Hence, we decide to examine the expression of NF-κB in our study. Previous report shows NF-κB activation is linked to the development of cardiac hypertrophy by Iso, and inhibition of NF-κB in cardiomyocytes is sufficient to impair Iso-induced hypertrophy (Freund et al., 2005).

In the present study, we examined the development of Iso-induced cardiac hypertrophy in cardiomyocytes following pretreatment with CGA. We also determined the status of NF-κB and reactive oxygen species (ROS) levels in cardiomyocytes following Iso or CGA treatment and explored the potential role of CGA in Iso-induced cardiac hypertrophy.

2. Materials and methods

2.1. Reagents

Isoproterenol and CGA were purchased from Sigma–Aldrich Chemical Co. Anti-cardiac troponin T (cTnT), which is a cardiac-specific capture antibody and labeled antibody (Koseki et al., 2010; Yamashita et al., 2005), was from Abcam. Anti-NF-κBIA was from ProteinTech Group Inc. Anti-ANP was from Gene Tex Inc. Anti-GAPDH was from Epitomics – an Abcam Company. Antibodies against NF-κB p65 (sc8008), PCNA (proliferating cell nuclear antigen) (sc56) were from Santa Cruz Biotechnology. Horseradish-peroxidase (HRP)-conjugated secondary antibodies and Cell lysis buffer were purchased from Beyotime Institute of Biotechnology. Bicinchoninic acid (BCA) Protein Quantitation Kit was from Pierce, Thermo Fisher Scientific Inc. The SuperSignal Substrate Chemiluminescence Kit was purchased from Millipore Corporation. Real-time polymerase chain reaction (PCR) primers were synthesized by Life Technologies Corporation. Reactive Oxygen Species Assay Kit was purchased from Beyotime Institute of Biotechnology. All other chemicals used in the experiment were of analytical grade.

2.2. Primary culture of neonatal rat ventricular myocytes

We obtained ventricles from 1-day-old Sprague-Dawley rats and isolated cardiac myocytes through digestion. Neonatal rats were obtained from Shanghai Slac Laboratory Animal Co. Ltd. The protocol was approved by the Institutional Animal Care and Use Committee of Tongji University. Briefly, the left ventricle were cut into small pieces (1 mm^3) and digested by 0.25% trypsin (Gibco, USA) in 37 °C water bath at 100 rpm for 10 min with a magnetic stirrer (Thermo, USA). The supernatant was discarded. The remaining tissues were further digested by 0.25% trypsin. The cell suspension was centrifuged at 90 rpm in 37 °C water for 8 min and the supernatant was resuspended in PBS (Wisent, Canada) containing 15% fetal bovine serum (FBS, Wisent, Canada). Thus repeatedly, all the supernatant in FBS were filtered into a 10 cm petri dish by 200 mesh membrane filter. Cells were cultured for 2 h to allow fibroblast cells to attach to the dish. Cardiomyocytes were collected from the supernatants and cultured with Dulbecco's modified Eagle's medium (DMEM, Wisent, Canada) containing 1 g/L glucose plus 10% FBS and 1% penicillin/streptomycin (Gibco, USA).

2.3. Cell culture and experimental treatments

Cardiomyocytes were grown in 2 ml DMEM containing 1 g/L glucose supplemented with 10% fetal bovine serum in six-well plates under a humidified atmosphere of 95% air-5% CO_2 at 37 °C. Cardiomyocytes were divided into five groups: (1) control group: given PBS in 2 ml DMEM complete medium as placebo for 2 h, and then 2 ml DMEM complete medium for 48 h; (2) Iso group: given PBS in 2 ml DMEM complete medium as placebo for 2 h, and then 10 μM Iso in 2 ml DMEM complete medium for 48 h; (3) low-dose CGA group: given 10 μM CGA in 2 ml DMEM complete medium for 2 h, and then 10 μM Iso in 2 ml DMEM complete medium for 48 h; (4) middle-dose CGA group: given 50 μM CGA in 2 ml DMEM complete medium for 2 h, and then 10 μM Iso in 2 ml DMEM complete medium for 48 h; and (5) high-dose CGA group: given 100 μM CGA in 2 ml DMEM complete medium for 2 h, and then 10 μM Iso in 2 ml DMEM complete medium for 48 h.

2.4. Immunofluorescence identification of cardiomyocytes isolated

Cultured cardiomyocytes were washed by PBS twice and fixed in 4% paraformaldehyde solution (Sangon Biotech, China) at room temperature for 20 min. After fixation, cardiomyocytes were washed by PBS to remove the fixative, and soaked in 0.5% (v/v) Triton-X-100 (Sigma, USA) in PBS for permeability. Five minutes later, cardiomyocytes were washed by PBST (0.5% Tween in PBS) three times, and soaked in 5% BSA (Sigma–Aldrich, USA) solution (g/mL, in PBS) for 1 h at 37 °C. Cardiomyocytes were washed twice in PBST and the primary antibody solution was added (1:100 dilution of anti-cTnT antibody in 2% BSA (g/mL, in PBS)). The antibody was covered with parafilm and incubated overnight at 4 °C. Primary antibody was removed with three washes of PBST. Subsequently, a fluorochrome-conjugated secondary antibody (Cy3) at a dilution of 1:200 in 2% BSA was added, incubated at room temperature for 1 h in darkness. The secondary antibody was removed with three 5 min washes in PBST followed by DAPI solution (PBS with 0.5 μg/mL 4,6-diamidino-2-phenylindole) staining at room temperature in darkness for 20 min. The stain solution was removed and washed by PBST. Antifade mounting medium was added prior to observing cardiomyocytes. Cell imaging was scanned under a fluorescence microscope (Olympus, Japan).

Identification of cardiomyocytes was also certified by morphological observation. Neonatal rat myocytes cultured in vitro show irregular shapes, such as spindle, angle and astrocytic. The most important feature is that myocardial cells can beat. Isolated cardiomyocytes, cultured for 48 h, were observed under the light microscope (Nikon Imaging Sales Co., Ltd., Japan).

2.5. Cytotoxicity of Iso and CGA on cardiomyocytes

The effects of Iso and CGA on cell viability were determined by testing the reducing enzymes present in viable cells to convert 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to formazan crystals. In brief, a solution of MTT from Sigma (5 mg/mL) was prepared in PBS, pH 7.2. The MTT solution was filtered through 0.22 μm syringe-driven filter (Merck Millipore Ltd.). Iso with increasing concentration (1, 5, 10, 15, 20, 25 μM) and CGA with 1, 5, 10, 50, 100, 150, 200 μM were added to the 96-well plates containing adhesive cardiomyocytes, 6 parallel groups of each concentration, respectively. After the plates were incubated in a CO_2 incubator at 37 °C, the medium in each well of the plate was discarded, and PBS washed each well twice. 20 μL MTT solution was added to each well of the 96-well plate containing 100 μL medium. The plates were incubated in a CO_2 incubator at 37 °C for 4 h. The medium was decanted off from the plate. 150 μL dimethyl sulfoxide (DMSO, Sangon Biotech Co., Ltd.) was added to each well on the micro oscillator (Kylin-Bell Lab Instruments Co., Ltd.) to dissolve the violet crystals, and the plates were shaken for 10 min. The absorbance in each well was read by a Thermo Scientific Multiskan FC (Finland) with 490 nm light wavelength.

2.6. Measurement of cell surface area

To study the protective effect of CGA against Iso-induced hypertrophy, Cells were plated at six-well plates. The cell grouping and culture were performed as mentioned above. The pretreated cells were washed by PBS twice prior to fixation in 4% paraformaldehyde solution. 10 min later, the residual paraformaldehyde solutions were removed with three 5 min washes of 0.1% Triton-X-100 (v/v, in PBS). Actin-Tracker Green at a dilution of 1:100 in diluent (PBS with 2% BSA and 0.1% Triton-X-100) was added. The six-well plates were incubated at room temperature for 30–60 min in darkness. Excess Actin-Tracker Green solutions were removed with four 5 min washes of 0.1% Triton-X-100 (see above) prior to visualizing cells under a Nikon inverted microscope equipped with a Polaroid digital camera at 200× magnification. Five random photographs were taken from each well and twenty-five individual cell surface areas were measured by ImageJ software (National Institutes of Health, USA).

2.7. Extraction of total RNA and reverse transcriptase polymerase chain reaction (RT-PCR)

The effect of GCA on the hypertrophic response of cardiomyocytes to Iso stimulus was studied by monitoring the reactivation of ANP, BNP and β-MHC by real

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