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Alpha-lipoic acid attenuates cardiac hypertrophy via inhibition of C/EBPβ activation

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ABSTRACT

Alpha-lipoic acid (ALA), a naturally occurring compound, exerts powerful protective effects in numerous cardiovascular disease models. However, the pharmacological property of ALA on cardiac hypertrophy has not been well investigated. The present study was carried out to determine whether ALA exerts a direct anti-hypertrophic effect in cultured cardiomyocytes and whether it modifies the hypertrophic process *in vivo*. Furthermore, we determined the potential underlying mechanisms for these actions. Treatment of cardiomyocytes with phenylephrine (PE) for 24 h produced a marked hypertrophic effect as evidenced by significantly increased in ANF and BNP mRNA levels, as well as cell surface area. These effects were attenuated by ALA in a concentration-dependent manner with a complete inhibition of hypertrophy at a concentration of 100 μg/mL. PE-induced cardiomyocyte hypertrophy was associated with increased mRNA and protein levels of C/EBPβ, which were inhibited by pretreatment with ALA. However, when cardiomyocytes were co-transfected with C/EBPβ, ALA failed to inhibit hypertrophic responses. Upregulation of C/EBPβ expression was also evident in rats subjected to 4 weeks of coronary artery ligation (CAL). However, rats treated with ALA demonstrated markedly reduced hemodynamic and hypertrophic responses, which were accompanied by attenuation of upregulation of C/EBPβ. Taken together, our results revealed a robust anti-hypertrophic and anti-remodeling effect of ALA, which is mediated by inhibition of C/EBPβ activation.

1. Introduction

Heart failure is a major global health problem and a leading cause of mortality (Zhang et al., 2013). In response to physiological, mechanical or pathological stress, the heart undergoes hypertrophic growth in what is thought to be an initial compensatory mechanism that temporarily maintains pump function (Hill and Olson, 2008). Unremitted hypertrophic growth is associated with adverse consequences that often lead to heart failure and sudden death (Seidman and Seidman, 2001).

The CCAAT/enhancer binding proteins (C/EBP) are a family of basic leucine zipper transcription factors (Nerlov, 2007). Previous study demonstrated that C/EBPβ represses cardiomyocyte growth and proliferation and that the reduction in C/EBPβ is a central signal in physiologic hypertrophy and proliferation (Boström et al., 2010). We recently reported that C/EBPβ plays a pivotal role in the pathogenesis of cardiac hypertrophy, and knockdown of C/EBPβ significantly attenuated PE-induced hypertrophic responses (Zou et al., 2014). Thus, better understanding of the signaling pathways affecting C/EBPβ expression, or drugs that inhibit C/EBPβ transcriptional activity in the heart, could be of significant clinical value in attenuating cardiac hypertrophy.

Alpha-lipoic acid (ALA) is a naturally occurring compound which can be found in food such as broccoli, spinach, and tomatoes (Deng et al., 2013). Collective evidence confirmed that ALA is involved in mitochondrial dehydrogenase reactions and has recently gained a considerable amount of attention as a novel antioxidant (Li et al., 2012). ALA has also been regarded as a therapeutic agent for a number of conditions related to cardiovascular disease, including atherosclerosis (Xu et al., 2012), diabetic cardiomyopathy (Hegazy et al., 2013), cardiac fibrosis (Lee et al., 2012). Dietary supplementation with ALA has been successfully employed in a variety of *in vivo* models: ischemia-reperfusion, heart failure, and hypertension (Ghibu et al., 2009). Besides, it has been reported that ALA treatment inhibited cyclosporine-induced hypertension and cardiac hypertrophy in SHR rats (Louhelainen et al., 2006). However, the

Abbreviations: ALA, alpha-lipoic acid; C/EBPβ, CCAAT/enhancer binding protein β; PE, phenylephrine; ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; CAL, coronary artery ligation; AngII, angiotensin II; ET-1, endothelin-1.

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effect of ALA against cardiac hypertrophy has not been well elucidated.

The present study is aimed to investigate the anti-hypertrophic effect of ALA using cultured ventricular myocytes and an *in vivo* model of cardiac hypertrophy secondary to chronic ischemia, and to clarify whether or not the underlying mechanism is due to the inhibition of C/EBPβ activation. Our results may shed new light on the understanding of the cardioprotective effect of ALA, and provide a new therapeutic strategy for the prevention and treatment of cardiac hypertrophy.

2. Materials and methods

2.1. Chemicals and reagents

Rabbit polyclonal antibody against C/EBPβ was purchased from Cell Signaling Technology (Danvers, MA, USA). GAPDH antibody and ALA were purchased from Sigma-Aldrich (St. Louis, MO, USA). NFATc4 antibody was purchase from Santa Crua Biotechnoloty (Santa Cruz, CA, USA). FLAG-C/EBPβ plasmid was acquired from Sangon (Shanghai, China). Horseradish peroxidase-conjugated secondary antibodies were acquired from Promega Co. (Madison, WI, USA). Polyvinylidene difluoride membrane was purchased from Immobilon®-PSQ (Millipore, Billerica, MA, USA). Lipofectamine™ 2000 reagent and Lipofectamine® LTX & Plus reagent were purchased from Invitrogen Molecular Probes, Inc. (Eugene, OR, USA). SYBR Premix Ex Taq II and MightyAmp® DNA Polymerase Ver.2 were obtained from TaKaRa Biotechnology Co., Ltd. (Dalian, China).

2.2. Coronary artery ligation

Following a 3-days acclimation period, a total of 40 Sprague-Dawley (male, weighing 250–300 g) rats were randomly assigned to either sham or coronary artery ligation (CAL) group ($n = 10$) without or with ALA treatment (200 mg/kg/ per day, i.p.) started 1 week after surgery and maintained for a further 4 weeks. CAL was performed as previously described (Chen et al., 2004). Briefly, rats were anesthetized with pentobarbital sodium (i.p, 50 mg/kg) before thoracotomy. The left main coronary artery was ligated 1–2 mm from its origin using a firmly tied silk suture. Sham-operated rats under a similar procedure without binding. All experimental procedures were carried out in compliance with the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (USA).

2.3. Echocardiographic analysis

Four weeks after CAL, rats were anesthetized with 2% isoflurane (Inhalation). Transthoracic echocardiography was performed with Vevo 2100 high-resolution *in vivo* micro-imaging system equipped with a real-time micro-visualization scan head of 16 MHz (Visual Sonics, Toronto, ON, Canada) as described previously (Harada et al., 1998). After a good-quality two-dimensional image was obtained, M-mode images of the left ventricle were recorded. Left ventricular internal diameter during diastole or systole (LVID), and anterior and posterior wall thickness during diastole or systole (AWT and PWT) were measured. All images were analyzed using the Vevo 2100 Protocol-Based Measurements software.

2.4. Hemodynamic and histological analysis

Hemodynamic measurement was carried out in all rats after anesthetized with pentobarbital sodium (i.p, 50 mg/kg). The right carotid artery was cannulated with a 24-gauge polyethylene catheter filled with heparin into the left ventricle. The left ventricular end-diastolic pressure (LVEDP) and left ventricular end-systolic pressure (LVESP), left ventricular end-diastolic pressure (LVESV) and left ventricular end-diastolic volume (LVEDV), the maximal rate of left ventricular pressure increase ($+dp/dt_{max}$) and decrease ($-dp/dt_{max}$) were recorded and analyzed by hemodynamic data analysis software (Tai-Meng, Chengdu, China).

For histological analysis, all the hearts were arrested in diastole with KCl (30 mmol/L), followed by perfusion fixation with 10% formalin. Fixed hearts were embedded in paraffin, sectioned at 4-μm thickness, and stained with hematoxylin and eosin for overall morphology. Mean myocyte diameter was calculated by randomly measuring 100 cells from sections stained with hematoxylin and eosin.

2.5. Neonatal cardiomyocytes culture and treatment protocol

Primary culture of neonatal cardiomyocytes were prepared from the left ventricles of 1- to 2-day-old SD rats as described previously (Stastna and Van Eyk, 2013). In brief, cardiomyocytes were cultured for 24 h in serum containing medium followed by 24 h in serum-free medium. To initiate hypertrophy, cells were then treated with 10 μM α_1 agonist phenylephrine (PE) for 24 h in the absence or presence of ALA (25, 50, or 100 μg/mL). For C/EBPβ overexpression, cardiomyocytes were transiently transfected with FLAG-C/EBPβ plasmid using Lipofectamine 2000 according to the manufacturer's instructions. The mRNA and protein levels of C/EBPβ in myocytes were shown in Supplementary Fig. S1A and B.

2.6. Measurement of cell surface area

Rhodamine-phalloidin (Invitrogen) was employed to visualize actin fragment in this assay. Cardiomyocytes grown in 48-well plates were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, followed by 0.5% Triton-100 treatment for 5 min. After incubated with 0.1% rhodamine-phalloidin for 30 min, cells were washed in PBS for further interaction with DAPI. The images of cardiomyocytes were detected by High Content Screening (ArrayScanVTI, Thermo Fisher Scientific, Rockford, IL, USA), and the cell surface area from randomly selected fields (50 for each group, at least 200 cells) was determined by the built-in image analysis software.

2.7. RNA isolation and quantitative RT-PCR (q RT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instruction, and subjected to a quantitative reverse transcription and polymerase chain reaction (RT-PCR) analysis with primers specific for atrial natriuretic factor (ANF), brain natriuretic peptide (BNP) and C/EBPβ, GAPDH were used as an internal standard. The following primer sequences were used: 5'-GGAAGTCAACCCGTCTCA-3' (forward) and 5'-AGCCCTCAGTTTGCTTTT-3' (backward) for ANF; 5'-TTTGGGCAGAAG ATAGACCG-3' (forward) and 5'-AGAAGAGCCGCAGGCAGAG-3' (backward) for BNP; 5'-AAGATGCGCAACCTGGAGAC-3' (forward) and 5'-CCTTCTTCTGCAGCCGCTC-3' (backward) for C/EBPβ; 5'-AGGAGTAA GAAACCCTGGAC-3'(forward)and 5'-CTGGGATGGAATTGTGAG-3' (backward) for GAPDH. The amplification conditions were 15 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 1 min at 55 °C and 30 s at 72 °C. All primers used were synthesized by Invitrogen and well-validated.

2.8. Western blot

Cultured cardiomyocytes and LV tissues were lysed in RIPA buffer containing 50 mM Tris (PH 7.4), 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS and 1 mM EDTA supplemented with protease inhibitor cocktail (Sigma, St. Louis, MO, USA) and 1 mM