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Candesartan cilexetil attenuated cardiac remodeling by improving expression and function of mitofusin 2 in SHR

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ABSTRACT

Background: Left ventricular hypotrophy (LVH) is very common in hypertensives even after antihypertensive treatment. Mitofusin 2 (Mfn2) is a critical negative regulator of vascular smooth muscle cell (VSMC) hypertrophy by regulating mitochondrial fusion, ras/raf/MEK signal pathway, et al. The purpose of this study was to investigate whether candesartan attenuated cardiac remodeling by improving expression and function of mitofusin 2 in SHR.
Methods: Nine weeks old spontaneously hypertensive rats (SHR) were selected and treated with candesartan for eight weeks. Then, heart tissues were investigated for signs of cardiac remodeling, mitochondrial structure and membrane potential, mitochondrial enzyme activities, hydrogen peroxide, mRNA and protein expression of Mfn2/ras/raf/MEK signaling pathway in heart tissues.
Results: The results showed that cardiac remodeling was obviously in SHR group: cardiac cell alignment was irregular; cardiac fibers became thick, irregular and enlarged; cell density was reduced in SHR compared to WKY. After candesartan treatment, histopathological structure improved significantly which were consistent with mitochondrial morphology, mitochondrial membrane potential, mitochondrial enzyme activities, hydrogen peroxide, Mfn2/ras/raf/MEK gene and protein expression in cardiac tissues. What's more, although blood pressure was well controlled in a normal range, cardiac remodeling wasn't avoided. In general, candesartan obviously repressed cardiac hypertrophy and cardiac remodeling significantly compared to SHR untreated group, but didn't reverse it.
Conclusions: Mfn2 is negatively associated with cardiac remodeling. Candesartan treatment can improve mitochondrial structure and function and regulate Mfn2/ras/raf/MEK signaling pathway. Mfn2 may be used a potential marker for cardiac remodeling and a novel therapeutic target for target organ damage protection.

1. Introduction

Cardiac hypertrophy can be defined as an increase in heart mass. Pathological hypertrophy is associated with increased interstitial fibrosis, cell death and cardiac dysfunction [1]. It is a response of hemodynamic overload in a disease setting, e.g. hypertension and valvular heart disease. Myocardial hypertrophy, especially left ventricular hypertrophy (LVH), is prevalent in a substantial portion of individuals with essential hypertension (EH) [2], and is a hallmark of cardiovascular disease and an independent risk factor for heart failure (HF) [3,4].

LVH is very common in both untreated hypertensive patients and patients treated with antihypertensive drugs, even those treated with newly developed AT1 antagonists [5]. A meta-analysis of more than 100 studies yielded a moderately strong relationship between BP reduction and LVM regression [6]. Recently, another meta-analysis reported that long-term and strict BP reduction by valsartan reversed LVH in 12.5% of EH patients [7], which meant 87.5% of EH patients may develop LVH and HF at last.

The molecular mechanism of pathological cardiac hypertrophy is involved of abnormalities of ras/raf/MEK, mitochondrial oxidative stress, calcium channels (ICa), Ang II AT1 receptor, apoptosis signaling pathway, calcineurin, and so on [8–10].

Mitofusin 2 (Mfn2, also named hyperplasia suppressive gene, HSG, NG_007945) is a new gene located at the mitochondrial outer membrane. It belongs to the family of large GTP-binding proteins and is located on the short (p) arm of chromosome 1 at position 36.22. Mfn2 is widely expressed in heart, VSMCs, brain and other tissues in rats as well as in human being [11,12]. Up-regulate Mfn2 expression can promote mitochondrial fusion [13]. The contribution of dysfunctional mitochondria to

Abbreviations: LVH, left ventricular hypertrophy; VSMC, vascular smooth muscle cell; EH, essential hypertension; Mfn2, mitofusin 2; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto rat; Δψm, mitochondrial membrane potential; ROS, reactive oxygen species; BW, body weight; HW, heart weight; SOD, superoxide dismutase; ARB, angiotensin II Type 1 receptor blocker; TOD, target organ damage.

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the pathogenesis of cardiovascular injury associated hypertension is also relevant [14,15], which supports an association between mitochondrial damage and hypertension. In spontaneously hypertensive rats (SHR), mitochondrial dysfunction is indicated by the occurrence of alterations in three aspects: ATP production [16]; reactive oxygen species (ROS) production and antioxidant defense levels [17,18]; and cytochrome oxidase activities and decreases in inorganic phosphate translocator [19,20]. What's more, Mfn2 is a critical negative regulator of vascular smooth muscle cell (VSMC) hypertrophy and proliferation by repressing of ras/raf/MEK pathway, a very important signaling pathway of VSMC hypertrophy and proliferation [12]. Besides pleiotropic non-fusion roles of mitofusin2, cardiac-specific deletion of Mfn2 has been reported to impair cellular autophagy which is evidenced by the accumulation of autophagosomes [21]. Lu Fang et al. [22] found that Mfn2 down-regulated in cardiac hypertrophy of SHR, pressure-overload hypertrophy by transverse aortic constriction, hypertrophy of non-infarcted myocardium and cardiomyopathy. Up regulation of Mfn2 inhibits Ang II-induced down regulation of Mfn2 gene expression and increases protein synthesis and hypertrophy in cardiomyocytes [23]. Therefore, Mfn2 can regulate mitochondrial fusion, oxidative stress, calcium infusion, ras/raf/ERK signal transduction, and apoptosis by different pathways, which are very important mechanisms of cardiac hypertrophy.

Despite considerable attention paid on cardiac hypertrophy and antihypertensive treatment, the roles of Mfn2 in evaluation and regulation of cardiac hypertrophy and remodeling in high blood pressure are still lacking. In this study, we aim to investigate if candesartan cilexetil treatment can attenuate cardiac remodeling and improving expression and function of mitofusin 2 in SHR, which will give a propose for Mfn2 as a potential target for cardiac hypertrophy prevention.

2. Methods

2.1. Animals, treatment and sample collection

Eight-week-old male WKY rats and SHR were obtained from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). Body weights of the animals were between 210 ± 10 g. Animals were housed in standard temperature (23 ± 2 °C) and light-controlled (12:12 h light–dark cycle) animal quarters. The protocol was reviewed and approved by the Animal Studies Committee of Beijing Anzhen hospital, and all experiments were performed in accordance with the National Institutes of Health guidelines for the use of experimental animals.

Twenty seven SHR were randomly divided into three groups: (1) positive control SHR group, SHR group, n = 9; (2) SHR + candesartan cilexetil (Canc) (2 mg/kg/day), SHR-Canc group, n = 9; and negative control WKY, WKY group, n = 9. All animals were allowed ad libitum access to standard rodent chow. Beginning at 9 weeks of age, rats were treated with different antihypertensive drugs dissolved in water and administered intragastrically daily [12]. The dose of drug administration was regulated depending on the body weight and blood pressure changes in the rat subjects. After 8 weeks of treatment, animals were killed by decapitation, and hearts were isolated for further analysis.

2.2. Blood pressure and heart weight measurement

Body weight (BW) and blood pressure were determined every week during the course of the experiment. Blood pressure (BP) was determined by the tail cuff method with a Photo Transistor (Muromachi MK2000ST, Japan), a non-invasive blood pressure monitors system. The record and analysis software came from the system itself.

The heart was rapidly excised and blotted dry; the great vessels were removed; and the heart weight (HW), composed of atria, ventricles, and septum, was recorded. Total heart weight–to–body weight ratio was used to express the degree of cardiac hypertrophy.

2.3. Histomorphology and immunohistochemistry of hearts

In brief, heart samples were isolated, fixed, dehydrated and embedded in paraffin. Sections (5 μm thick) were stained with hematoxylin–eosin.

Following citrate antigen retrieval (BioGenex), paraffin sections were blocked for one hour at room temperature in a blocking solution (PBS with 0.1% cold water fish skin gelatin, 1% bovine serum albumin, 0.1% Tween-20, and 0.05% NaN_3) and was used to dilute antibodies. First antibodies were incubated overnight at 4 °C, second antibodies for 2 h at room temperature; PBS was used for washes [24]. Sections were examined under light microscopy with an attached video camera.

2.4. Ultrastructure of hearts

The heart was isolated and fixed quickly in 2.5% glutaric dialdehyde for 2 h at 4 °C. After washing with phosphate buffer (pH 7.4), the sample was post-fixed in 1% osmium tetroxide for 2 h at 4 °C. Gradient dehydration by 50% to 100% ethanol was performed, and the sample was then embedded in epoxies at 37 °C overnight. Ultrathin sections were cut at 50 nm, and stained with lead citrate solution. Then, transmission electronic microscope was used for observing the structural changes of hearts [25].

2.5. Isolation of membrane fraction and mitochondria

After being excised and rinsed, hearts were immediately minced and suspended in a digestion medium containing 0.3 M sucrose, 1 mM $CaCl_2$, 5 mM MOPS, 0.1% (wt/vol) BSA, collagenase 70 mg/mL and 5 mM KH_2PO_4 (pH 7.4). The mixture was incubated at 0 °C for 40 min. Ethylene Glycol Tetraacetic Acid (EGTA) at a final concentration of 2 mM was added to stop the incubation. The digestion medium was discarded and replaced with fresh medium containing 0.3 M sucrose, 1 mM EGTA, 5 mM MOPS, 0.1% (wt/vol) BSA, 5 mM KH_2PO_4 (pH 7.4). The tissue was homogenized at 4 °C [26].

After centrifugation of the homogenates at 1500 g for 10 min, the supernatant was centrifuged at 10,000 g for 10 min. The ensuing crude mitochondrial fraction was washed once and then resuspended in 0.23 M mannitol, 0.07 M sucrose, 1 mM EDTA in 10 mM Tris–HCl (pH 7.4). To evaluate the activity of mitochondrial NOS (mtNOS), the mitochondrial fraction was further purified by suspension in 30% (vol/vol) Percoll in 0.25 M sucrose, 1 mM EDTA, 0.1% (wt/vol) BSA in 10 mM Tris–HCl (pH 7.4) and centrifugation at 90,000 g for 30 min. Purified mitochondria were washed twice with 150 mM KCl, followed by two washes with 0.23 M mannitol, 0.07 M sucrose, 1 mM EDTA in 10 mM Tris–HCl (pH 7.4) [27].

2.6. Detection of mitochondrial membrane potential ($\Delta\psi m$)

About 50 mg of left ventricular tissue was isolated. Then, the samples were washed twice with 4 mL of PBS, cut into small pieces, and incubated in trypsin/PBS solution on ice for 3 min. Then, we isolated mitochondria from these samples using the mitochondria isolation kit for Tissue (Pierce Biotechnology, Inc.) following the kit's instructions [28].

$\Delta\psi m$ was estimated by using JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazolyl carbocyanine iodide) to visualize the state of mitochondrial membrane potential. The transition from potential dependent loss of J-aggregates (hyperpolarization) to accumulation of JC-1 monomers (depolarization) is represented by a fluorescence emission shift from red to green, as visualized by confocal microscopy. The mitochondrial membrane potential assay kit (Beyotime Biotech, China) was used according to the manufacturer's protocol. The fluorescence changes were then analyzed for JC-1 uptake by using Zeiss LSM510 META confocal laser scanning microscope (Zeiss, Thornwood, NY) excited at 485 nm (for JC-1) and set to simultaneously detect green emissions (510 nm) and red emissions (590 nm) channels. The