Renal denervation attenuates cardiac hypertrophy in spontaneously hypertensive rats via regulation of autophagy

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Received July 26, 2016; Accepted June 8, 2017

DOI: 10.3892/mmr.2017.6790

Abstract. It has been suggested that renal denervation (RD) may attenuate left ventricular (LV) hypertrophy. However, the role that autophagy serves in this process is currently unclear. In the present study, utilizing a model of hypertension-induced cardiac hypertrophy in spontaneous hypertensive rats, it was demonstrated that RD was significantly associated with a reduction in LV hypertrophy. Furthermore, a decrease in the myocardial mRNA of hypertrophy-associated genes was demonstrated in RD rats compared with sham controls. In addition, RD in hypertension-induced LV hypertrophy rats was associated with the attenuation of cellular autophagic response over activation at a physiological level. This was indicated by a reduction in the expression of Beclin-1, autophagy related 9A and microtubule-associated protein 1A and 1B-light chain 3 II/I in RD rats to physiological levels that are observed in control rats. Furthermore, the number of autophagosomes was restored to physiological levels in the cardiomyocytes of RD rats. The results of the current study suggest that RD may attenuate LV hypertrophy via the regulation of autophagic responses.

Introduction

Despite significant advances in recent decades in diagnosis and treatment, congestive heart failure (CHF) remains one of the most prevalent cardiovascular diseases and contributes to morbidity and mortality in both developing and developed countries (1,2). The pathogenesis of CHF is poorly understood, although it was initially thought that exposure to harmful stimuli led to damage of cardiomyocytes (3,4). Activation of the sympathetic neural system, renin-angiotensin-aldosterone system, and inflammatory pathways act as compensatory mechanisms in the acute stage, thereby resulting in cardiac hypertrophy. However, chronic over-activation of these pathways leads to pathological ventricular remodeling resulting in deterioration in cardiac function (3,4). Therefore, uncovering the potential mechanisms and signaling pathways underlying the transition from adaptive cardiac changes to maladaptive pathological cardiac hypertrophy is of important significance for the development novel treatments, as well as aiding in preventative strategies for CHF.

Recent evidence from both clinical and animal studies has indicated that autophagy, a conserved cellular process responsible for bulk degradation and recycling of cytoplasmic components such as proteins and organelles, may play an important role during the pathogenesis of cardiac hypertrophy (5-7). Physiologically, a basal level of autophagy is fundamental for maintaining the homeostasis of cardiomyocytes by timely degradation of cytoplasmic components (8,9). However, defects in basal autophagic activity and over activation of autophagic responses can lead to disturbance of cellular homeostasis and contribute to the initiation of processes that lead to apoptosis (10). Therefore, regulation and manipulation of myocardial autophagic activity has become a novel target for the prevention and treatment of cardiac hypertrophy and subsequent CHF.

Hypertension, which directly leads to pressure overload of the left ventricle (LV), has been recognized as an important cause of both cardiac hypertrophy and CHF. Correspondingly, many antihypertensive medications, such as the angiotensin II converting enzyme inhibitors (ACEIs), angiotensin II receptor inhibitors (ARBs) and β adrenaline receptor blockers have the ability to attenuate the pathophysiologic processes of cardiac hypertrophy, alongside their antihypertensive effects (11-13). Recent clinical trials have demonstrated that renal sympathetic denervation (RD), a newly emerging treatment strategy for patients with resistant hypertension, may also ameliorate the...
development of cardiac hypertrophy (14-16). Indeed, an early study in 46 patients with hypertension demonstrated that RD significantly reduces LV mass and improves diastolic function at 6-month follow-up (17). These observations have been confirmed in subsequent clinical studies (18-20). Interestingly, some animal studies also found similar benefits of RD on cardiac hypertrophy and cardiac remodeling in various animal models (21-23). Mechanisms aside from attenuation of blood pressure and reduction in the activity of sympathetic nerves have been proposed to account for these changes, including a reduction in myocardial inflammation (22). However, whether RD has the ability to attenuate cardiac hypertrophy, induced by pressure overload of the LV, remains to be confirmed, and the potential mechanisms by which this may occur are not currently known.

Therefore, in this study with a previously well-established model of hypertension induced LV hypertrophy in spontaneous hypertensive rats (SHR), we aimed to determine whether RD attenuated cardiac hypertrophy. More importantly, as the disturbance of autophagic activity has been implicated in the pathogenesis of cardiac hypertrophy, we aimed to investigate whether regulation of cardiac autophagy responses play a potential role underlying the benefits of RD against LV hypertrophy. Results of our study may be of value in understanding the potential mechanisms underlying the benefits of RD for cardiac hypertrophy, and may provide further evidence for the clinical applications of RD in the prevention and treatment of CHF.

Materials and methods

Animal grouping and renal denervation procedure. Spontaneously hypertensive and renal denervation procedure. Spontaneously hypertensive rats (SHRs, n=16) and aged matched Wistar-Kyoto (WKY, n=6) rats weighing 240-280 g were purchased from Vitalriver Co., Ltd. (Beijing, China). Briefly, rats were kept in a room with constant temperature of 26°C, humidity of 55%, and a 12 h light/dark cycle throughout the study. Animals were given standard rat chow and tap water ad libitum. Specifically, the SHRs were randomly divided into 2 groups, the renal denervation group (RD, n=8) and the sham-operated group (Sham, n=8). Age matched WKY rats served as negative controls (NC, n=6). RD surgery was performed on rats aged 12 weeks or younger, as previously described (24). Briefly, the rats were anesthetized with choral hydrate (3 ml/kg, ip). The parameters of left ventricular wall thickness, heart chamber sizes, and ejection fraction (EF) of each rat were recorded using a transthoracic echocardiography (Philips IE33; Philips Healthcare, DA Best, The Netherlands) with a 7.5 MHz sector scan probe. The following parameters were measured from M-mode traces: Left ventricular posterior wall diastolic and end-systolic thicknesses (LVPWd and LVPVs), interventricular septal end-diastolic and end-systolic dimensions (IVSd and IVSs), EF and heart rate (HR).

Histological analyses. After the echocardiographic examination, rats were sacrificed, and hearts from rats in each group were excised. The heart weight (HW) and the body weight (BW) were obtained subsequently, and the ratio of HW to BW (HWI) was calculated. Samples of left ventricles were fixed with 10% neutral buffered formalin and embedded with liquid paraffin. Other myocardial tissue samples were fixed in 4% formaldehyde and embedded in paraffin. These tissues were then sectioned at 6 μm and stained with hematoxylin and eosin (H&E). Cell surface area (CSA) of cardiomyocytes was then measured and analyzed with NIS-Elements F 4.0 using a fluorescence microscope (Nikon, Eclipse, Ti-U; Nikon Corporation, Tokyo, Japan). Five random fields from each of 4 sections per animal were analyzed, and 10-15 cardiomyocytes per section were measured. The quantification of diameter and area of cardiomyocytes were determined with Image Pro Plus 6.0 (Media Cybernetics, Carlsbad, CA, USA).

Quantitative PCR (qPCR) for the analyses of hypertrophy related genes. Total cellular RNA was isolated from tissues using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA) and cDNA was synthesized using a PrimeScript II 1st Strand cDNA Synthesis kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturers’ protocols. qPCR was then performed using the SYBR® Premix Ex Taq™ II Kit (Takara Bio, Inc.) in a LightCycler®480 II (Roche, Basel, Switzerland). We used 18S rRNA as internal controls for mRNA detection. The relative mRNA levels of ANP and β-MHC were calculated using the 2-ΔΔCq method as previously described (25). The primer sequences are shown in Table I.
Western blot analysis. Total cellular protein was extracted from cardiomyocytes using NP-40 lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). Protein concentrations were then assessed by a Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After, equal amounts of protein samples (40 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane. For Western blotting, the PVDF membrane was blocked with 5% skimmed milk powder for 1 h and then incubated with a rabbit polyclonal antibody against autophagy-related proteins (Cell Signaling Technology, Inc., Danvers, MA, USA), a rabbit monoclonal antibody against GAPDH (Epitomics, Burlingame, CA, USA) at 4°C overnight. The next day, membranes were washed with phosphate-buffered saline-Tween-20 (PBS-T) thrice and then further incubated with an anti-rabbit peroxidase-conjugated secondary antibody (Boiworld, Wuhan, China). The immunological complexes were then visualized by chemiluminescence BeyoECL Plus (Beyotime Institute of Biotechnology). Protein expression was normalized to levels of GAPDH.

Transmission electron microscopy (TEM) examination. Myocardial cell samples were processed as previously described (26) and TEM examination was performed using a Tacnai 12 Spirit Twin transmission electron microscope at a magnification of x13,500. For each cell section, 10 images were taken randomly from different fields to calculate the number of autophagic vacuoles by an experienced investigator of TEM who was blinded to the origin of each image. The morphological criteria of autophagosomes or autolysosomes were set as followings in accordance with the current consensus (27): At the ultrastructural level, a double-membrane structure containing undigested cytoplasmic contents, which had not fused with lysosomes, or intracellular organelles such as mitochondria, and fragments of the endoplasmic reticulum (ER).

Statistical analysis. Experimental data were expressed as mean ± standard error (SEM). The Shapiro-Wilk test was used to assess whether the data followed a normal distribution. For comparison between two groups, a Student's t test was performed, while for comparison between more than two groups, an ANOVA was used. All tests were two-sided and P<0.05 was considered to indicate a statistically significant difference. Statistical analyses were performed using SPSS 11.0 (SPSS, Inc., Chicago, IL, USA).

Ethics statement. All animal experimental protocols were performed following the Guidelines of Council for International Organization of Medical Sciences (CIOMS) and were approved by the review board of the Animal Care and Ethics Committee of Guangzhou Medical University.

Results

Effects of RD on cardiac structure, function and blood pressure in SHRs. Results of previous studies indicated that cardiac hypertrophy develops spontaneously in SHRs arising from increased pressure overload induced by hypertension (28). As shown in Table II, results of our study confirmed these results by showing that the dimensions of LVPW and IVS in both end-systolic and diastolic phases of rats from the sham group were significantly increased compared with rats from the NC group 4 weeks after the surgical procedures (P<0.05), with significantly increased SBP and DBP levels (P<0.05). However, no significant differences were detected in EF and HR between the 2 groups. Subsequently, we found that RD significantly decreased SBP and DBP levels in RD group, compared with those from the sham group. Further, dimensions of LVPW and IVS in rats from RD groups were also significantly decreased (P<0.05) as compared to sham group, suggesting that RD treatment may attenuate myocardial hypertrophy induced by pressure overload.

Effects of RD on pathological cardiac hypertrophy in SHRs. To further confirm the effect of RD treatment on pathological myocardial hypertrophy in SHRs, we observed the histological changes of myocardium, HWI, and CSAs of cardiomyocytes among the rats from the 3 groups. Images of whole LV cross-sections showed that the LV volume was remarkably reduced, while the papillary muscles and trabeculae carneae coridis were much coarser in appearance in the sham group than in the NC group (Fig. 1A), which was consistent with the finding that HWI was also increased in sham rats compared with NC rats (Fig. 1; P<0.05). We further confirmed that increased HWI in sham rats was accompanied with significantly increased CSAs of cardiomyocytes (Fig. 1B). Interestingly, the rats from the RD groups showed significantly reduced HWI and CSAs of the cardiomyocytes, compared with rats from the sham group 4 weeks after RD treatment, further suggesting that RD treatment was associated with attenuated myocardial hypertrophy induced by pressure overload. However, RD was not associated with improved cardiac function in our study as evidenced by LVEF in echocardiographic study. We hypothesize that in our present study we only focus on the effect of RD treatment in the early cardiac hypertrophy, maybe the significant result of improving cardiac function was acquired by extending RD-treated time.

Effect of RD on the expression of hypertrophy-related gene mRNA in LV tissues. Myocardial hypertrophy has been associated with upregulated expression of fetal myocardial genes, including ANP and β-MHC (29). Therefore, we observed whether RD treatment could reverse these changes...
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Table II. Effects of RD on cardiac structure, function and hemodynamic parameters evaluated by echocardiography.

|                | NC        | Sham     | RD        |
|----------------|-----------|----------|-----------|
| IVSd (10⁻³ cm) | 122.83±5.71| 194.25±9.23⁺ | 138.42±6.34⁻ |
| IVSs (10⁻³ cm) | 216.52±16.43| 348.13±13.32⁺ | 236.27±11.46⁻ |
| LVPWd (10⁻³ cm) | 153.17±8.61 | 217.38±12.52⁺ | 168.41±9.87⁻ |
| LVPWs (10⁻³ cm) | 216.34±6.79 | 321.25±8.24⁺ | 224.68±4.73⁻ |
| EF (%)         | 89.70±1.89 | 86.38±0.82 | 88.24±1.26  |
| HR (bpm)       | 362.14±43.02| 352.13±41.82 | 335.67±58.10 |
| SBP (mmHg)     | 142.21±9.68 | 201.28±8.63⁺ | 166.35±22.91⁻ |
| DBP (mmHg)     | 76.50±6.32 | 148.54±10.36⁺ | 92.62±6.25⁻  |

Data were expressed as mean ± SEM; n=6-8 in each group;⁺P<0.05 compared to the NC group;⁻P<0.05 compared to the sham group. RD, renal denervation; IVSd, interventricular septal end-diastolic dimension; IVSs, interventricular septal end-systolic dimensions; LVPWd, left ventricular posterior wall end-diastolic thickness; LVPWs, left ventricular posterior wall end-systolic thickness; EF, ejection fraction; HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Effect of RD on the expression of autophagy-related proteins in LV tissues. Since changes in myocardial autophagy has been implicated in the development of cardiac hypertrophy (10,30), we went on to examine whether the beneficial effects of RD procedure on cardiac hypertrophy in SHRs was associated with the regulation of autophagy response, by evaluating the changes of autophagy-related proteins (i.e., Beclin-1 and ATG9A is marked as autophagic formation and LC3 and p62 is referenced as autophagic flux) during the pathophysiological process.

Our data showed that the levels of Beclin-1, ATG9A and LC3II/I were significantly increased in the sham rats compared with NC rats, but the p62 was significantly decreased in the sham rats compared with NC rats, all of above indicating a hyperactive autophagic response during the pressure overload induced cardiac hypertrophy (Fig. 3). Interestingly, treatment with RD significantly reduced the myocardial expressions of Beclin-1, ATG9A and LC3II/I, but significantly improved at a molecular level. As shown in Fig. 2, gene expression of ANP and β-MHC was induced in rats from sham group as compared with those from NC group 4 weeks after surgical procedure. Moreover, RD treatment significantly reduced the myocardial level of expression of these 2 hypertrophy related genes (Fig. 2; P<0.05), confirming that RD treatment could suppress cardiac hypertrophy at molecular level.
the myocardial expression of p62 (Fig. 3), suggesting that RD treatment in SHRs may attenuate cardiac hypertrophy via alleviation of the hyperactive cardiac autophagic response.

**Effect of RD on the number of autophagic vacuoles in LV tissues.** To further confirm the changes of myocardial autophagic response during the pathophysiological process, we evaluated the changes of myocardial autophagic vacuoles under TEM. The data showed that compared with NC rats, the number of autophagic vacuole in cardiomyocytes in sham rats was significantly increased (Fig. 4; P<0.05), reflecting a hyperactive myocardial autophagic response during the pathogenesis of pressure overload induced cardiac hypertrophy in SHRs. Moreover, we found that the number of autophagic vacuole in cardiomyocytes from RD rats was significantly reduced in comparison to the sham group, further confirming that RD treatment in SHRs attenuates the cardiac hypertrophy via alleviation of the overactivated cardiac autophagic response.

**Discussion**

In this animal experiment, by using a well characterized model of pressure overload induced cardiac hypertrophy in SHRs, we confirmed previous findings that hyperactive myocardial autophagy plays an important role in the pathogenesis of pressure overload induced cardiac hypertrophy as shown by induction of autophagy-related proteins (i.e., Beclin-1, ATG9A, LC3 and p62), and formation of autophagosomes in cardiomyocytes of SHRs. Moreover, consistent with previous findings in clinical and experimental observations, we found that bilateral RD in SHRs attenuated the progression of pressure overload induced cardiac hypertrophy, as evidenced by the reduced heart weight to body weight ratio, reduced thicknesses of LV walls via echocardiography, decreased CSAs of cardiomyocytes, and decreased mRNA levels of fetal genes related to cardiac hypertrophy in RD rats. Moreover, bilateral RD in SHRs also alleviated the hyperactive myocardial autophagic response at a physiologic level as seen in NC rats. To the best of our knowledge, our study is the first to report the potential benefits of RD against hypertension induced LV hypertrophy, and the associated attenuation of overactivated cellular autophagic reaction. Results of our study may be helpful for understanding the potential mechanisms underlying the beneficial effects of RD on LV hypertrophy, and provide further rationale for evaluation of the clinical use of RD in the prevention LV hypertrophy in patients with hypertension.

Previous evidence in clinical studies has strongly suggested the potential benefits of RD in the attenuation of...
LV hypertrophy (17,19). Another interesting finding retrieved from previous clinical observations is that the regression of LV mass following bilateral RD seems to occur independently from the effects of blood pressure lowering and potential pathophysiological mechanisms underlying the preventative effect of RD on LV hypertrophy and CHF deserve further investigation (18,19). Results of previous animal experiments also support the protective role of RD against LV hypertrophy. In a previous study with SHRs, Jiang et al found that RD can significantly delay the progression of left ventricular hypertrophy, possibly not only through the suppression of sympathetic activity and attenuation of pressure load, but also due to the reduction in myocardial inflammation, as reflected by significantly reduced myocardial levels of Toll like receptor 4 (TLR4), nuclear factor kappa B (NF-κB), tumor necrotic factor alpha (TNF-α) and interleukin 6 (IL-6) after RD (22,31). In a subsequent study in hypertensive type 1 diabetic rats, Thaung et al found that bilateral RD reduces cardiac hypertrophic remodeling in these rats without restoring the attenuated cardiac β-AR responsiveness (32). Instead, they proposed that indirect cardiac effects, such as attenuation of sympathetic innervation of the systemic vasculature and/or kidney may be involved (32). Results of our study are consistent with these observations by showing that bilateral RD may effectively attenuate pressure overload induced LV hypertrophy by showing changes in both the cellular morphology and myocardial expression of fetal genes related to cardiac hypertrophy. Our results, together with the findings of above studies, confirmed the potential protective role of RD on LV hypertrophy, and these benefits seem to be dependent on multiple mechanisms other than decreases in blood pressure.

Researches within the last decade have indicated that autophagy, a conserved cellular response for bulk degradation and recycling of cytoplasmic components, may participate in the pathogenesis of many cardiovascular disorders, including LV hypertrophy and CHF (5,6). Previous evidence from in vitro and in vivo studies has suggested that autophagy maintained at physiological levels is necessary for intracellular homeostasis of cardiomyocytes (7). During the pathogenesis of cardiac hypertrophy and CHF, the autophagy response in the myocardium may have dual roles (33). Adaptive and well-controlled autophagy may be considered a survival mechanism, as it may be helpful for efficiently degrading overproduced cytoplasmic components, formed during LV hypertrophy. Conversely, uncontrolled and hyperactive autophagy may lead to degradation and damage to the necessary cytoplasmic components, and finally lead to apoptosis and necrosis of the cardiomyocytes, thereby accelerating the progression of LV hypertrophy or even CHF (34). Interestingly, results of our mechanical studies showed that during the pathogenesis of LVH in rats from sham group, the autophagic response was hyperactive, as reflected by the significant induction of autophagy specific proteins and increased autophagosomes in the myocardium of sham compared with NC rats, which confirmed previous findings. Moreover, we found that bilateral RD in SHRs alleviated the overactivation of cellular autophagic response induced by pressure overload, and restored the activity of myocardial autophagy to a similar extent of physiological level, as in NC rats, reflected by the similar levels of myocardial autophagy specific proteins and numbers of autophagosomes of RD and NC rats. These results suggest, for the first time to the best of our knowledge, the beneficial effects of bilateral RD against pressure overload induced cardiac hypertrophy, and may be related to the attenuation of the hyperactive myocardial autophagic response at an adaptive physiological level. Clearly, the signaling pathways underlying the regulation effects of RD on myocardial autophagic response deserve further investigation.

In conclusion, results from our study suggested that RD may attenuate LV hypertrophy via regulation of autophagic responses, which may provide further evidence for the clinical applications of RD in the prevention of LV hypertrophy.
Acknowledgements

This study was supported by the Research Funding of Doctoral Program of Guangzhou Medical University (Grants no. 2014C37) and the Doctoral Program of Guangdong Natural Science Foundation (Grants no. 2015A030310068).

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