Mechanical Stress Triggers Cardiomyocyte Autophagy through Angiotensin II Type 1 Receptor-Mediated p38MAP Kinase Independently of Angiotensin II

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Abstract

Angiotensin II (Ang II) type 1 (AT1) receptor is known to mediate a variety of physiological actions of Ang II including autophagy. However, the role of AT1 receptor in cardiomyocyte autophagy triggered by mechanical stress still remains elusive. The aim of this study was therefore to examine whether and how AT1 receptor participates in cardiomyocyte autophagy induced by mechanical stresses. A 48-hour mechanical stretch and a 4-week transverse aorta constriction (TAC) were imposed to cultured cardiomyocytes of neonatal rats and adult male C57BL/6 mice, respectively, to induce cardiomyocyte hypertrophy prior to the assessment of cardiomyocyte autophagy using LC3b-II. Losartan, an AT1 receptor blocker, but not PD123319, the AT2 inhibitor, was found to significantly reduce mechanical stretch-induced LC3b-II upregulation. Moreover, inhibition of p38MAP kinase attenuated not only mechanical stretch-induced cardiomyocyte hypertrophy but also autophagy. To the contrary, inhibition of ERK and JNK suppressed cardiac hypertrophy but not autophagy. Intriguingly, mechanical stretch-induced autophagy was significantly inhibited by Losartan in the absence of Ang II. Taken together, our results indicate that mechanical stress triggers cardiomyocyte autophagy through AT1 receptor-mediated activation of p38MAP kinase independently of Ang II.

Introduction

Under physiological conditions, cardiac hypertrophy serves as a compensatory response to increased hemodynamic load, including hypertension [1], vascular disease [2], and myocardial infarction [3]. However, sustained or excessive hypertrophic responses may prompt transition to decompensated pathological hypertrophy en route to ultimate heart failure [4]. The definite irreversible cellular sequelae for decompensated cardiac hypertrophy is cardiomyocyte death, as evidenced by loss of cardiomyocyte histologically [5]. Three distinct modes of cell death have been demonstrated including necrosis (oncosis), apoptosis, and autophagy [6]. Necrosis and apoptosis invariable contribute to cell death, whereas autophagy may serve as a double-edged sword to provide both pro-survival and pro-death roles. In patients with heart failure, autophagy was found to be excessive, much more than that of apoptosis, correlating with left ventricular systolic dysfunction [6,7].

Autophagy is a highly conserved catabolic cellular process for protein and nutrient recycling. Initially described as a cellular survival mechanism in starvation, autophagy has recently gained much attention as a mechanism for cell death in a number of diseases including heart failure [8]. Excessive autophagy has been documented in myocardial damages, contributing to the onset and progression of pathological conditions such as pressure overload-induced cardiac remodeling and heart failure [9,10,11]. Angiotensin II (AngII) and its type 1 receptor (AT1) have been reported to play a pivotal role in the etiology of heart failure possibly related to autophagy [12]. Nonetheless, the precise role of AT1 receptor and autophagy, if any, in pressure overload-induced heart failure remains elusive. Moreover, the mitogen-activated protein kinase (MAPK) family such as ERKs, JNK and p38MAP kinase have been implicated in pressure overload-induced cardiac hypertrophy [13,14]. Nonetheless, whether these stress signaling molecules are involved in cardiomyocyte autophagy remains elusive. To this end, the present study was designed to examine the role of AT1 receptor in pressure overload-induced cardiomyocyte autophagy en route to heart failure, and the role of ERKs, JNK and p38MAP kinase in AT1 receptor-mediated myocardial autophagy responses, if any.

Methods

Animal models

All animal procedures were approved by the Animal Care and Use Committee of Fudan University and were in compliance...
Figure 1. Involvement of AT_1 receptor in mechanical stretch-induced cardiomyocyte hypertrophic and autophagic responses. Cultured cardiomyocytes of neonatal rats were treated with vehicle (Control), stretch in the absence or presence of Losartan (10^(-6) mol/L) or PD123319 (10^(-6) mol/L); A: [3H]-Leucine incorporation in cardiomyocytes; B: Cardiomyocyte morphology and size; cardiomyocytes were subjected to
with the Guidelines for the Care and Use of Laboratory Animals published by the National Academy Press (NIH Publication No. 85-23, revised in 1996). In brief, C57/B six male mice, aged 8-10 weeks, were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) and were subjected to transverse aorta constriction (TAC) or sham operation under anesthesia (ketamine, 25 mg/kg, i.p.) as described [15]. Following anesthetization and artificial ventilation, the transverse aorta was constricted with the 7.0 nylon suture by ligating the aorta together using a blunted 27-gauge needle. The needle was pulled out immediately after the procedure. Losartan (3 mg/kg/day, Sigma-Aldrich), SB203580 (10 μmol/L), or PD123319 (10 μmol/L), or SP600125 (10 μmol/L) was pre-administered using Alzet osmotic minipumps (Model 2002, DURECT, Cupertino) implanted subcutaneously into the back of mice 3 days prior to TAC. Four weeks later, all mice were sacrificed and hearts were excised for further examination.

Echocardiography and haemodynamic measurements

Transathoracic echocardiography was performed using 30MHz high frequency scanner (Vevo770, VisualSonics Inc. Toronto, Canada) [16]. All measurements, averaged for five consecutive cardiac cycles, were carried out by three experienced technicians unaware of experimental group identity. Blood pressure (BP) was evaluated as described [17,18]. A micromanometer catheter (Millar 1.4F, SPR 835, Millar Instruments, Inc., Houston, TX) was inserted into the right common carotid artery, while the transducer was connected to a Power Laboratory system (AD Instruments, Castle Hill, Australia) in order to record BP.

Morphology and histological Analyses

Excised hearts were weighed, perfused with PBS and fixed with 4% polyformaldehyde for global morphometry and then with 10% formalin for further histological analysis. Paraformaldehyde-fixed hearts were sectioned at 4-μm thickness and stained with hematoxylin and eosin (H-E). Cardiomyocyte morphology and histology was visualized under a high magnification to assess cross-sectional area (CSA) using a video camera (Leica Qwin 3) attached to a micrometer. Twenty randomly chosen fields were evaluated from each cross section of the left ventricle (LV) free wall.

Cell culture and treatment

Primary cultured cardiomyocytes derived from neonatal rats [19](Shanghai Institutes for Biological Sciences, Shanghai, China) and COS7 cells [20](Shanghai Institutes for Biological Sciences, Shanghai, China) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS). The cells were then incubated in serum and antibiotic-free conditions in silicon-based plates pre-coated for 24 hours with collagen prior to application of 48-hour mechanical stretch to 120% [15]. Losartan (10 μmol/L, PD123319 (10 μmol/L, PD98059 (10 μmol/L), SB203580 (10 μmol/L), or SP600125 (10 μmol/L) was pre-administered in the culture medium for 30 min. The mechanical stretch last for 48 hours and cardiomyocytes were collected for extraction of protein and total RNA for further study. For transfection experiments, COS7 cells were incubated for 24 hours before transfection.

[3H] Leucine Incorporation

Cultured cardiomyocytes were incubated with [3H] leucine (1 μCi/ml) in silicon-based plates pre-coated with collagen. Cells were then subjected to a 48-hour mechanical stretch before being exposed with 5% trichloroacetic acid. Protein precipitates were dissolved in 1 ml of 100 mmol/L NaOH and radio activity was determined using a liquid scintillation counter.

Real-time RT-PCR

Total RNA was isolated from LV tissues or culture cells using TRIzol® reagent according to the manufacturer’s instruction. After purification, RNA was subjected to real-time RT-PCR analysis for the expression of atrial natriuretic peptide (ANP) and skeletal α-actin (Saa) on a BIO-RAD IQ5 multicolor detection system. The melting curves and quantization were analyzed using Light Cycler and Rel Quant software, respectively. A comparative CT method was used to determine relative quantification of RNA expression [21]. All PCR reactions were performed at least in triplicate.

Western blot analyses

Total proteins isolated from LV tissues or culture cells were detected for LC3b-II using western blot analysis. Cells were lysed before centrifuged at 200×g to remove the nuclei. The supernatant was centrifuged at 15,000×g for 30 min to collect cell membrane. Total proteins were size-fractionated by SDS-PAGE and were transferred to Immobilon-P membranes (Millipore). The blotted membrane were incubated with antibodies against LC3b-II (Cell Signaling Technology Inc. Beverly, MA, USA), and subjected to an ECL Detection system (GE healthcare).

Autophagic flux assessment

For autophagic flux assessment, cardiomyocytes were treated with vehicle or chloroquine (CQ, 20μmol/L) [22] for 4 hours and cell lysates were prepared for the detection of LC3b-II protein abundance.

Generation of GFP-LC3 vector and fluorescent microscopic analysis

A lentiviral vector containing GFP-LC3 reporter (GFP-LC3) was constructed. Cardiomyocytes were transfected with lentivirus particles (MOI = 20) and then were mechanical stretched for 48 hours. Cells were observed under fluorescent microscope.

Immunofluorescence

Following cell culture on silicon-based plates in serum-free DMEM for 48 hours, cardiomyocytes or COS7 cells were incubated with anti-α-MHC (Upstate, catalogue 05-716, USA) and LC3b-II (Cell Signaling Technology Inc. catalogue 2775, USA). The samples were then incubated with secondary antibodies conjugated with FITC or Alex (Invitrogen, catalogue...
A21206, USA) according to the manufacturer’s instructions. The surface areas of cardiomyocytes and LC3b-II were determined using an image analysis software (Leica Qwin 3) and were calculated using the mean of 100 to 120 cells from randomly selected fields.

Statistical analysis

Data are shown as means ± s.e.m. Comparison was performed by one-way analysis of variance followed by Newman-Keuls test for post-hoc analysis to determine the difference among the groups.

Results

1. Involvement of AT_1 receptor in mechanical stretch-induced cardiomyocyte hypertrophic and autophagic responses

Mechanical stretch-induced hypertrophic responses were manifested as increased cellular protein synthesis in neonatal cardio-

![Image of Figure 2](https://example.com/image2.png)

**Figure 2. Involvement of p38MAP kinase in mechanical stretch-induced cardiomyocyte autophagy.** A: Phosphorylation of ERKs, JNK and p38MAP kinase was examined using Western blotting; Total protein levels of ERKs, JNK and p38MAP kinase were used as the loading control. Representative photograms were shown from 3 independent experiments; B: Western blot analyses and immunofluorescent staining for LC3b-II using the anti LC3b-II antibody (red); β-actin in whole cell lysate was used as the loading control. Representative photograms from 3 independent experiments are shown.

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myocytes as evaluated by the [3H]-Leucine incorporation assay (Fig. 1A), enlarged cell surface area measured by immunofluorescence (Fig. 1B), and reprogramming of hypertrophy-associated fetal genes including \textit{Anp} and \textit{Saa} measured using quantitative real-time PCR (Fig. 1C). Interestingly, the mechanical stress-induced hypertrophic responses were significantly attenuated by treatment with the AT1 receptor antagonist Losartan (Fig. 1A–C). However, treatment with the AT2 receptor antagonist PD123319 failed to affect mechanical stretch-induced hypertrophic responses (Fig. 1A–C).

Cultured cardiomyocytes exhibit a significant increase in cardiomyocyte autophagy as evidenced by the increased LC3b-II following a 48-hour mechanical stretch (Fig. 1D). To examine whether mechanical stretch affects autophagic flux, we subjected cardiomyocytes with mechanical stretch in the absence and presence of chloroquine, which inhibits lysosomal acidification and prevents autophagosome-lysosome fusion. The control group demonstrated a basal level of cardiomyocyte autophagy, with autophagosome accumulation (<3-fold increase) in the presence of chloroquine, suggesting the intact autophagic flux. Mechanical stretch caused a 5.1-fold increase in autophagosome abundance compared with the control group, implying the induction of autophagosome formation (Fig. 1D). To confirm the characteristics of autophagy elicited by mechanical stress, puncta GFP-LC3 and accumulation of autophagosomes in cardiomyocytes were investigated by fluorescence microscopy. Fluorescence microscopy analysis showed that a punctate pattern of LC3 was observed in mechanical stress-treated cardiomyocytes, whereas a diffused distribution of LC3 was found in control cardiomyocytes (Fig. 1E). To ask whether AT1 or AT2 receptor was involved in mechanical stretch-induced cardiomyocyte autophagy, we used pharmacological inhibitors. Intriguingly, the AT1 receptor blocker Losartan, but not AT2 receptor inhibitor PD123319, drastically suppressed mechanical stretch-induced cardiomyocyte autophagy (Fig. 1F).

2. Involvement of p38MAP kinase in mechanical stretch-induced cardiomyocyte autophagy

To examine whether the MAPK family participates in mechanical stress-induced cardiac autophagy, phosphorylation of ERKs, JNK and p38MAP kinase were evaluated. Our results revealed significantly elevated phosphorylation of these stress signaling molecules in response to mechanical stretch (Fig. 2A). Although inhibition of ERKs, JNK and p38MAP kinase all ablated mechanical stress-induced cardiac hypertrophic responses, only p38 MAPK inhibitors but not ERK or JNK suppressed mechanical stretch-induced autophagic responses (Fig. 2B).

3. Mechanical stress-induced autophagy through AT1 receptor and p38MAP kinase independently of Ang II

To examine if mechanical stress induces autophagy through AT1 receptor independent of Ang II, we examined COS7 cells exposed to mechanical stress. Our data revealed that stimulated COS7 cells failed to induce a significant increase in autophagy (as evidenced by LC3b-II) in response to mechanical stress. However, they developed an increase in autophagy when transfected with AT1 receptor. Moreover, mechanical stress-induced autophagy was suppressed by p38MAP kinase inhibitor (Fig. 3).
However, the precise role of AT1 receptor and subsequently considered to mediate Ang II-induced cardiomyocyte autophagy reported that mechanical stress is capable of activating AT1 receptor-mediated p38MAP kinase independent of Ang II. Further investigation is warranted to better understand the molecular mechanism of AT1 receptor signaling during the pressure overload process.

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Author Contributions
Conceived and designed the experiments: LL JG YZ. Performed the experiments: LL XL CT YY WW LW. Analyzed the data: JX YY. Contributed reagents/materials/analysis tools: YY. Wrote the paper: LL JG YZ.

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