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Autophagy activation attenuates angiotensin II-induced cardiac fibrosis

Shenglan Liu [a, 1], Shaorui Chen [a, 1], Min Li [a], Boyu Zhang [a], Peiye Shen [a], Peiqing Liu [a, b], Dandan Zheng [a], Yijie Chen [a], Jianmin Jiang [a, *]

[a] Laboratory of Pharmacology and Toxicology, School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou 510006, PR China
[b] National and Local Joint Engineering Laboratory of Druggabilitiy Assessment and Evaluation, School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou 510006, PR China

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ABSTRACT

Autophagy has been involved in numerous diseases processes. However, little is known about the role of autophagy in cardiac fibrosis. Thus, whether or not angiotensin II (Ang II)-induced autophagy has a regulatory function on cardiac fibrosis was detected *in vitro* and *in vivo*. In rat cardiac fibroblasts (CFs) stimulated with Ang II, activated autophagy was observed using transmission electron microscopic analysis (TEM), immunofluorescence and Western blot. In Ang II-infused mice, increased co-localization of LC3 puncta with vimentin was observed. In rat CFs, co-treated with rapamycin (Rapa), an autophagy inducer, Ang II-induced the upregulation of type I collagen (Col-I), fibronectin (FN) was decreased. Conversely, inhibition of autophagy by chloroquine (CQ), an autophagy inhibitor, or knockdown of ATG5, a key component of the autophagy pathway by specific siRNA, aggravated Ang II-mediated the accumulation of Col-I and FN. Furthermore, in C57 BL/6 mice with Ang II infusion, intraperitoneal administration of Rapa ameliorated Ang II-induced cardiac fibrosis and cardiac dysfunction, while CQ treatment not only exacerbated Ang II-mediated cardiac fibrosis and cardiac dysfunction, but also impaired cardiac function. These findings suggest that autophagy may exert a protective role to attenuate excess extracellular matrix (ECM) accumulation in the heart.

1. Introduction

Cardiac fibrosis is a critical aspect of cardiac dysfunction following myocardial infarction, hypertension, heart failure and severe arrhythmia. Relentless production and progressive accumulation of extracellular matrix (ECM) proteins are hallmarks of cardiac fibrosis. During cardiac remodeling, cardiac fibroblasts (CFs) can differentiate to myofibroblasts, leading to synthesis and release various cytokines and deposition of ECM [1]. In the past decade, myocardial fibroblasts have been demonstrated to be greatly involved in myocardial development and in pathologies including cardiac fibrosis related diseases. Therefore, intervention of myocardial fibrosis is likely to be an effective strategy in curbing various heart diseases [2].

Macroautophagy, generally referred to as autophagy is a conserved, genetically regulated and ubiquitous pathway (from yeast to mammals) that promotes the turn-over of cell macromolecules and organelles via the lysosomal degradative pathway to maintain cellular homeostasis [3]. Upon initiation of autophagy, a small vesicular phagophore elongates and subsequently encloses a portion of cytoplasm, which results in formation of autophagosomes. In phagophore elongation, microtubule-associated protein light chain 3 (LC3-I) is lipidated, which can be detected as the change from LC3-I to LC3-II. LC3 is thus used as a marker for autophagosomes. Then the autophagosome fuses with a lysosome to form an autolysosome, leading to degradation of the enclosed

Abbreviations used: Ang II, angiotensin II; ECM, extracellular matrix; CFs, cardiac fibroblasts; Rapa, rapamycin; CQ, chloroquine; Col-I, type I collagen; FN, fibronectin; LAMP1, lysosomal associated membrane protein 1; TEM, transmission electron microscopy; ER, endoplasmic reticulum; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; TBS-T, Tris buffered saline-Tween 20; HRP, horseradish peroxidases; MMPs, matrix metalloproteinases; TGF, transforming growth factor.

* Corresponding author. School of Pharmaceutical Sciences, Sun Yat-sen University, 132 East, Waihuan Road, Guangzhou Higher Education Mega Center, Guangzhou 510006, PR China.
E-mail address: lssjjm@mail.sysu.edu.cn (J. Jiang).

[1] Shenglan Liu and Shaorui Chen contributed equally to this work.

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materials [4]. Another possible autophagic marker, Beclin-1, encoded by the beclin-1 gene, is the mammalian ortholog of yeast Atg6 that is required for the initiation of autophagy through its interaction with Vps34 [5].

Autophagy can be upregulated in response to both intracellular and external factors, such as growth factor withdrawal [6], acid starvation [7], endoplasmic reticulum (ER) stress [8], oxidative stress [9], hypoxia [10], organelle damage [11] and pathogen infection [12]. In the heart, basal autophagy occurs constitutively in the normal myocardium, but is strongly upregulated in cases of cardiac hypertrophy [13], heart failure [14], and ischemic cardiomyopathy [15]. Autophagy has been shown to play a protective role rather than a detrimental one in the heart [16,17]. Although increasing evidence has shown that dysregulated autophagy involved in disorders characterized by fibrosis in various tissues, including liver [18] and lung [19], little is known about the role of autophagy in cardiac fibrosis, which ultimately leads to various cardiac diseases.

In the present study, we aim to investigate the functional role of autophagy in the progression of cardiac fibrosis induced by Ang II. To address that issue, rat CFs treated with Ang II *in vitro* and the mice with subcutaneous Ang II infusion *in vivo* were used. Moreover, the effect of Ang II on the induction of autophagy and the role of agents that alter the autophagic process in the progression cardiac fibrosis were examined.

2. Materials and methods

2.1. Materials and regents

Rapamycin (Rapa) and chloroquine (CQ) were purchased from Selleck (Selleck Chemicals, Houston, TX, USA), Dulbecco's modified Eagle's medium (DMEM) and angiotensin II (Ang II) were from Merck (Merck Millipore, Billerica, MA, USA), fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Invitrogen/GIBCO (GIBCO, Life Technologies, Grand Island, NY, USA). Anti-LC3 and anti-vimentin antibodies were provided by Sigma–Aldrich (Sigma–Aldrich, St. Louis, MO, USA). Anti-Beclin-1 and anti-α-tubulin antibodies and all secondary antibodies were purchased from Cell Signaling Technology (Cell Signaling Technology, Boston, USA). Anti-ATG5 antibody was provided by Novus (Novus, Cambridge, UK). Anti-Col-I antibody was purchased from Abcam (Abcam, Cambridge, UK) and anti-FN antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Rat cardiac fibroblasts isolation and culture

Animals' handlings and the experimental procedures were conforming to the Guidelines of Animal Experiments from Ethical Committee for Animal Research of Sun Yat-sen University. Adult male rat CFs were isolated as described previously [20]. Briefly, the hearts were isolated from the adult male Sprague Dawley rats (180–220 g) and were minced, digested in 0.1% collagenase type II solution (GIBICO, Grand Island, NY, USA) at 37 °C for 30 min, 0.25% trypsin (Sigma–Aldrich, St. Louis, MO, USA) for three 5-min periods. After digestion, the cell suspension was centrifuged at 1200 rpm for 3 min and resuspended with DMEM plus 10% FBS, and then seeded in culture flask. After 1 h incubation, the debris and non-adherent cells were removed, and the attached cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C. The rat cardiac fibroblasts of second to fourth passages were used.

2.3. Cytotoxicity assay

Rat CFs were seeded in 96-wells plate (5000 per well) overnight before they were treated with Rapa or CQ of various concentrations for 24 h. Subsequently, cells were incubated with 3-(4, 5-dimethylthiazol-2-y1)-2, 5-dipheny-ltetrazolium bromide (MTT, 5 mg/mL, Sigma–Aldrich, St. Louis, MO, USA) for 4 h at 37 °C. Then the MTT medium was removed and 150 μL dimethylsulfoxide (DMSO, Sigma–Aldrich, St. Louis, MO, USA) was added to each well and agitated for 10 min. The absorbance was measured at a wavelength of 490 nm with an automated micro-plate reader (Bio-Tek, Winooski, VT, USA).

2.4. Immunofluorescence assay

Cells were fixed in 4% formaldehyde for 30 min, permeabilized with 1% Triton X-100 for 10 min, blocked with 10% normal goat serum for 30 min at room temperature, followed by overnight-incubation with anti-LC3 antibody (1:100 dilution) at 4 °C. Rat CFs were washed and incubated with Alexa Fluor-labeled secondary antibody (1:1000 dilution) for 1 h at room temperature. Washed with cold PBS three times to remove away the excess antibody, cells were stained with Hoechst 33342 for 10 min. Images were captured using a confocal microscope (Zeiss LSM 710). For quantification of the puncta formation, at least three optical fields with at least 30 cells per experimental condition were analyzed. Data from repeated experiments are subjected to statistical analysis.

Frozen sections of the left ventricles from mice were prepared in 10 μm thick sections and fixed 4% paraformaldehyde for 30 min, then washed with PBS buffer and permeabilized with 1% triton X-100 for 15 min, blocked, and incubated with antibodies to LC3 (1:200) and vimentin (1:200) overnight at 4 °C. Following washing with PBS, sections were incubated with fluorescently conjugated secondary antibodies. Slides were washed with PBS again and stained with DAPI. Images were captured using a confocal microscope (Zeiss LSM 710).

2.5. Transfection of cells with ATG5 siRNA

Duplex siRNAs specific for ATG5 and the negative control siRNA were synthesized and purchased from GenePharma (Shanghai, PR China). Rat CFs were transiently transfected with selected 100 nM of siRNA targeting to ATG5 (the oligo sequence was 5′-GACGCUGGUAACUGACAAATT-3′, GenePharma Co., Ltd, Shanghai, China) by using 5 μL of the siRNA transfection reagent (Lipofectamine 2000; Invitrogen; Life Technologies, Carlsbad, CA). Cells were also transfected with the control negative siRNA duplex. After 60 h of transfection, cells were treated with Ang II (10^{-7} mol/L) for 24 h. Then cells were harvested and whole-cell lysates were prepared after treatment. The effects of the ATG5-siRNA on the expression of endogenous ATG5 and LC3 were monitored at protein level by Western blot.

2.6. Animal experiments

Male C57 BL/6 mice weighing 20–25 g were supplied by the Experimental Animal Center of Sun Yet-sen University (Guangzhou, China). Then mice were randomly divided into four groups: the control group with vehicle-infused (n = 7), the Ang II-infused group (n = 7), the Rapa treatment group with intraperitoneal injection of Rapa at a dosage of 2 mg/kg/day for two weeks (n = 7), the CQ group with intraperitoneal injection of CQ at a dosage of 20 mg/kg/day for two weeks (n = 7). All of the groups, except the control group with vehicle-infused, simultaneously received Ang II (1.2 mg/kg/day) from an implanted subcutaneous minipump (ALZET micro-osmotic pump MODEL 1002 DURECT Co., Cupertino, CA) for two weeks, Mice were housed in temperature-controlled and humidity-