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Activation of calcium-sensing receptor-mediated autophagy in angiotensinII-induced cardiac fibrosis in vitro

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ABSTRACT

Cardiac fibrosis is one of the primary mechanisms of ventricular remodeling, and there is no effective method for reversal. Activation of calcium sensing receptor (CaSR) has been reported to be involved in the development of myocardial fibrosis, but the molecular mechanism for CaSR activation has not yet been clarified and needs to be further explored. Here, we found that AngII induces cardiac fibroblast proliferation and phenotypic transformation in a dose-dependent manner with increased CaSR and autophagy related protein (Beclin1, LC3B) expression. CaSR activation results in intracellular calcium release, MEK1/2 pathway phosphorylation, autophagy activation and collagen formation induced by AngII in cardiac fibroblasts. However, pretreating the cells with Calhex$_{231}$, PD98059 or 3-MA partially blocked AngII-induced cardiac fibrosis. Our data indicate that the activation of CaSR-mediated MEK/ERK and autophagic pathways is involved in AngII-induced cardiac fibrosis in vitro.

1. Introduction

Hypertensive heart disease seriously affects the patient's quality of life. Ventricular remodeling is the main pathological mechanism for this phenomenon [1]. In addition to compensatory hypertrophy of cardiomyocytes, abnormal proliferation and activation of fibroblasts plays an important role, leading to myocardial systolic dysfunction and heart failure. Currently, there is no effective method of reversing hypertensive myocardial fibrosis. Cardiovascular clinical and basic research has always focused on the pathogenesis and prevention of cardiac fibrosis.

The calcium-sensing receptor (CaSR) is a member of the superfamily of G protein-coupled receptors (GPCRs) [2]. Recent studies have found that CaSR is associated with many cardiovascular diseases, including myocardial ischemia-reperfusion injury, heart failure, and vascular calcification [3–5]. Zhang et al. first reported the expression of CaSR in cardiac fibroblasts and initially discussed the role of CaSR in Ca^{2+} channel-mediated fibroblast proliferation [6]. We have observed significant cardiac fibrosis and increased CaSR expression in spontaneously hypertensive rat myocardial tissue (in submission). However, the mechanism of cardiac fibrosis modulation by CaSR is not fully understood.

Autophagy is the primary metabolic process by which eukaryotic cells degrade and recover damaged macromolecules and organelles [7,8]. During this process, substances in the cytoplasm are phagocytosed by autophagosomes, which are spherical structures with double layer membranes and are transported to lysosomes for degradation. After binding to late endosomes or lysosomes, autophagosomes and their contents are degraded. The degradation products can be reused in the syntheses of macromolecules and in energetic metabolism [8]. Autophagy is a highly conserved catabolic process that appears to govern several cardiac pathologies [9,10]. Previous work indicates a potential balance between adaptive and maladaptive autophagic induction, and autophagy inhibitors may shed light on the role of autophagy in the associated pathological signaling processes [10,11]. Heart failure and autophagy studies have revealed autophagosomes within the myocardium in ischemia/reperfusion models [12,13]. Several studies have exploited autophagy inhibition in the diseased heart by using lysosomotropic agents, such as bafilomycin-A1 (Baf-A1) and chloroquine (CQ) [14,15]. In human atrial fibroblasts, TGF-β1 treatment caused parallel induction of fibrogenesis and autophagy [16]. Despite these findings, the possibility that autophagy promotes fibroblast activation and phenoconversion in unpassaged cardiac

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fibroblasts has not been explored.

AngiotensinII (AngII) plays an important role in the onset and development of cardiac remodeling [17]. Research showed that AngII could lead to myocardial remodeling and upregulate myocardial autophagy [18,19]. However, whether AngII leads to cardiac fibrosis through CaSR-mediated autophagy remains to be answered.

2. Materials and methods

2.1. Chemicals and reagents

Antibodies against CaSR were purchased from Alpha Diagnostic International Inc. (San Antonio, Texas, USA). Antibodies against MMP9, MMP2 and α-SMA were purchased from Abcam (Cambridge, UK). Antibodies against Beclin 1, LC3B, *p*-MEK1/2, *t*-MEK1/2, *p*-ERK1/2, *t*-ERK1/2 and GAPDH were purchased from Cell Signaling (Beverly, MA, USA). AngII, Calindol, $Calhex_{231}$, PD98059 and 3-MA was purchased from Sigma (Santa Clara, CA, USA).

2.2. Cell culture

Neonatal cardiac fibroblasts (CFs) were isolated from the hearts of 1- to 3-day-old Wistar rats. Briefly, the hearts were quickly excised and immediately embedded in PBS solution. CFs were isolated from the rat hearts by enzymatic digestion with pancreatin and grown to 80% confluence, then cultured in serum-free medium, exposed to hypoxia (95% N2, 5% CO2) and treated with AngII, Calindol or $Calhex_{231}$.

2.3. Viral infection

CFs were seeded at 210^5 cells/well in a confocal dish. The mature form of GFP-mCherry-LC3-expressing adenovirus at a multiplicity of infection (MOI) of 20 was allowed to adhere for 24 h according to manufacturer's protocol (Beyotime, Shanghai, China).

2.4. Electron microscopy

Cultured cardiac fibroblasts or ventricles of mice were subjected to the preparation of ultrathin sections. Cells were collected from the culture plate by brief trypsinization. Cell pellets were fixed with 4% paraformaldehyde and 1% glutaraldehyde in PBS overnight at 4 °C. Cells were then washed and dehydrated with increasing concentrations of ethanol. Ultrathin sections were prepared, followed by embedding and sectioning. Observation was carried out by a microscopist using a H7650 transmission electron microscope (Hitachi, Japan). For each treatment, the micrographs of at least 20 unique cells were recorded.

2.5. Western blotting analysis

Cells were washed twice with ice-cold PBS and incubated for 30 min in protein lysate containing the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). The cells were mechanically disrupted and centrifuged at 13,000 rpm for 15 min at 4 °C to remove nuclei. The protein concentration of the supernatant was determined with a Bradford protein assay, using bovine serum albumin (BSA) as the standard. The proteins (30 μg) were subjected to SDS-PAGE (10% (w/v) acrylamide gel) and blotted onto a nitrocellulose membrane in 39 mM glycine, 48 mM Tris (pH 8.3), 20% (v/v) methanol, and 10% SDS. Membranes were blocked at 37 °C for 1 h in TBST (137 mM NaCl, 20 mM Tris (pH 7.5), and 0.1% (v/v) Tween 20) containing 5% (w/v) skimmed milk. The membranes were then incubated overnight at 4 °C with antibodies anti-CaSR (1:500), anti-Beclin 1(1:1000), anti-LC3B (1:1000), anti-P62(1:1000), anti-*p*-

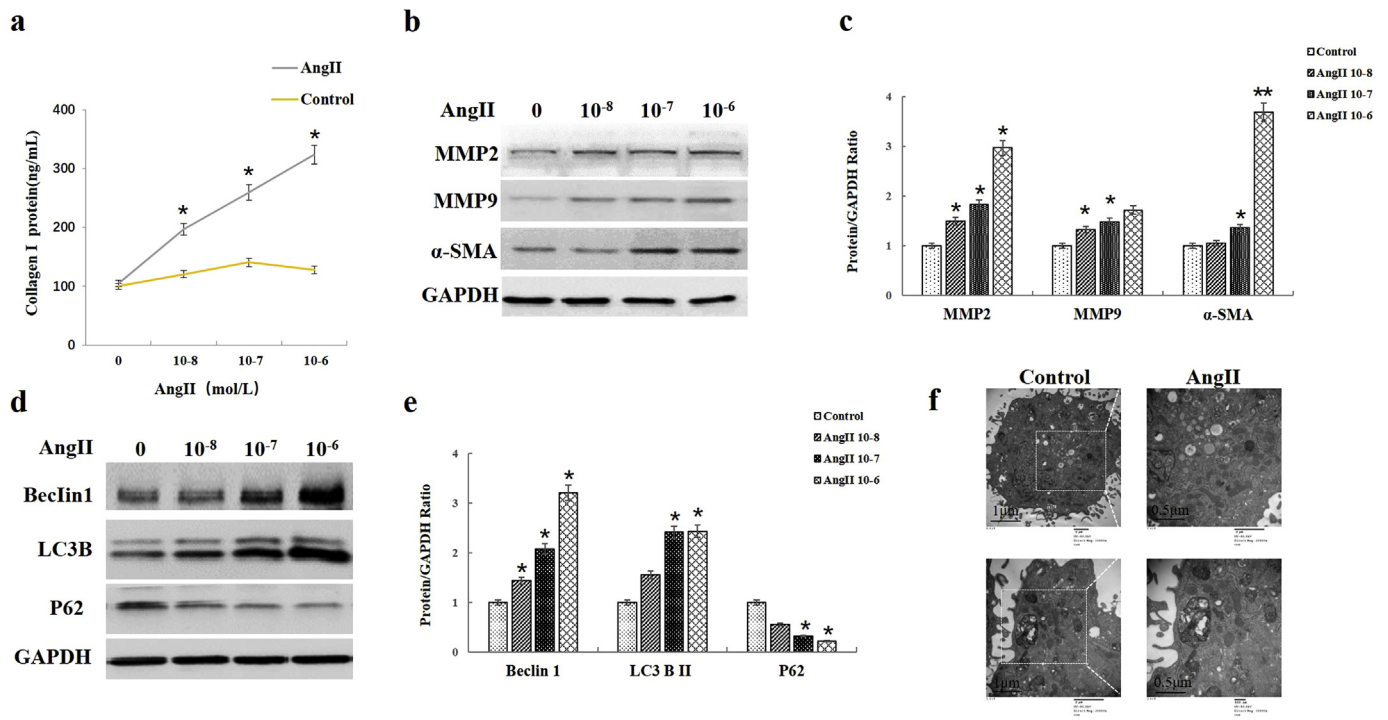

Fig. 1. AngII induces cardiac fibroblast proliferation and phenotypic transformation (a) CFs cultured in vitro were treated with 10^{-8}, 10^{-7}, and 10^{-6} mol/L AngII for 24 h. We used an ELISA kit to detect collagen I expression in the supernatant. (b–e) CFs were treated with 10^{-8}, 10^{-7}, and 10^{-6} mol/L AngII for 24 h, and MMP2/9, α-SMA, Beclin 1, LC3B II and P62 protein were observed. n = 3. This experiment was repeated three times. *$p < 0.05$ as calculated by the Student's *t*-test, when compared to values in control cells. (f) Cardiac fibroblasts were treated with AngII (10^{-7} mol/L) for 24 h. Cells were subjected to transmission electron microscopy. Note the presence of double-membraned autophagosomes.

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