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MiR-208a-3p aggravates autophagy through the PDCD4-ATG5 pathway in Ang II-induced H9c2 cardiomyoblasts

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ABSTRACT

Pathological cardiac hypertrophy is the main determinant of the development of heart failure, for which there is often no effective therapy. The dysregulation of autophagy is implicated in hypertrophy, but the mechanism linking these processes is unclear. In this study, we characterized the regulatory role of miR-208a-3p in autophagy in H9c2 cardiomyoblasts induced by Angiotensin II (Ang II). We found that miR-208a-3p was up-regulated in Ang II-induced H9c2 cardiomyoblasts and in starvation-induced autophagy. The overexpression of miR-208a-3p increased Ang II-induced autophagy, and this was accompanied by the inhibition of programmed cell death protein (PDCD4) and upregulation of autophagy protein 5 (ATG5). A dual-luciferase report assay confirmed the direct binding between miR-208a-3p and PDCD4. PDCD4 knockdown up-regulated autophagy, and its overexpression down-regulated this process. Moreover, the PDCD4-mediated regulation of autophagy was modulated by ATG5. Taken together, these findings indicate that miR-208a-3p promotes autophagy during Ang II-induced hypertrophy and provide a basis for the development of therapies for hypertrophic-induced cardiac dysfunction.

1. Introduction

Cardiac hypertrophy is a major process in cardiac adaption to hemodynamic overload or contractile failure of the myocardium. A long period of cardiac hypertrophy can result in contractile dysfunction, cardiac decompensation, and even heart failure [1]. Pathologically, cardiac hypertrophy is often accompanied by fibrosis, capillary rarefaction, increased production of proinflammatory cytokines, and cellular dysfunction [2]. Cardiomyocyte hypertrophy is a complex process characterized by reversion to fetal gene expression patterns, the transformation of various pathways, and cytoskeletal changes [3–5].

Autophagy maintains cardiac homeostasis, but can be damaging when it is not carefully regulated. Autophagy is important for clearing damaged cells and organelles, which is crucial for proper heart function, but excessive autophagic activity may prompt heart failure [6]. Therefore, therapies that regulate autophagy may be beneficial for hypertrophic-induced cardiac dysfunction.

MicroRNAs (miRNAs), a family of endogenous, conserved, small non-coding RNAs, regulate gene expression by binding to the 3′-untranslated regions (3′-UTRs) of target mRNAs to elicit the degradation or translational inhibition of target genes [7]. They are key regulators of complex biological processes linked to multiple cardiovascular pathologies. For example, the miR-212/132 family, miR-199a, and miR-221 are important regulators of autophagy and hypertrophy [8–10]. Nevertheless, until recently, only a few miRNAs involved in cardiac autophagy have been discovered, and little is known about how they regulate autophagy and hypertrophy. The miRNA family miR-208 includes two members, miR-208a and miR-208b, with similar nucleotide sequences [11]. miR-208a is specifically expressed in the heart and influences the muscle myosin content and muscle performance [11]; miR-208a-3p is the mature sequence of miR-208a and has been linked to hypertrophy. Van Rooij showed that the inhibition of miR-208a ameliorates cardiac function and improves survival during heart failure [12]. However, the role of miR-208a-3p in autophagy and its molecular mechanism are currently unknown.

PDCD4, a programmed cell death factor, is a target of miR-21, a tumor-associated miRNA [13]. PDCD4 has been implicated in cell growth and apoptosis, but its specific role is unclear [14]. A miR-208a-3p binding site was detected in the 3′-UTR of the *PDCD4* gene using a bioinformatics approach, suggesting that miR-208a-3p regulates PDCD4 expression and is involved in the pathological process of heart disease.

In the present study, we investigated the molecular role of miR-208a-3p in autophagy and its relevant molecular mechanisms. Using an in vitro model, we showed that miR-208a-3p overexpression modulates autophagy induced by Angiotensin II (Ang II) in H9c2 cardiomyoblasts

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by inhibiting PDCD4 and up-regulating ATG5. PDCD4 was identified as a direct target of miR-208a-3p. Furthermore, we showed that PDCD4 can inhibit ATG5 and thus impair autophagy.

2. Materials and methods

2.1. Cell culture and drug administration

H9c2, an embryonic rat myocardium-derived cell line, was obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM; Corning Inc., NY, USA) and 10% fetal bovine serum (FBS; Corning Inc., NY, USA) at 37 °C in 5% CO_2. The H9c2 cardiomyoblasts were exposed to 150 nM recombinant human Ang II (Sigma-Aldrich, St. Louis, MO, USA), which served as hypertrophic agonists. To induce autophagy by starvation, the culture medium was replaced with Earle's balanced salt solution (EBSS; Thermo Fisher Scientific, Waltham, MA, USA) for 4 h in H9c2 cardiomyoblasts.

2.2. RNA extraction and real-time quantitative polymerase chain reaction (RT-PCR)

The cells were lysed using TRIzol reagent (TaKaRa, Dalian, China), and the extracted RNA samples were reverse transcribed into cDNA using PrimeScript RT Master Mix (TaKaRa). PCR amplification was performed on an ABI 7300 thermocycler (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex-Taq (TaKaRa). For miRNA, cDNA was synthesized from miRNA with the Mir-X miRNA First-Strand Synthesis kit and PCR amplification used Mir-X miRNA qRT-PCR SYBR Kit (TaKaRa). The expression levels of PDCD4, ATG5, BNP, and β-MHC mRNA were normalized to an endogenous control (GAPDH). MiR-208a-3p levels were normalized to its control, U6, and fold-changes were calculated using the 2ΔΔCt method. The following specific primer sequences were used: (from 5′ to 3′): rat BNP: CAGAAGCTGCTGGAGCT GATAAG and TGTAGGGCCTTGGTCCTTTG; β-MHC: GGCTGGCTACAG AAGAACAAG and TACAGGTGCATCAGCTCCAG; PDCD4: ATGAGACG GCGTTTGAGAAG and AGGCTAAGGACACTGCCAAC; ATG5: TATCAG AGCATGTCACCCTT and TTCCTGTCTGGCTTGCAGCA; GAPDH: GGCA CAGTCAAGGCTGAGAATG and ATGGTGGTGAAGACGCCAGTA; and miR-208a-3p: CATAAGACGAGCAAAAAGCAAA.

2.3. Protein extraction and immunoblotting analysis

Cells were lysed by RIPA lysis buffer supplemented with 1% phenylmethylsulfonyl fluoride (Beyotime, Shanghai, China). Equal amounts of protein were separated on a SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). Primary antibodies against LC3B (Novus Biologicals, Littleton, CO, USA), P62 (Proteintech, Wuhan, China), PDCD4 (Proteintech, Wuhan, China), ATG5 (Cell Signaling Technology, Danvers, MA, USA), and GAPDH (Proteintech, Wuhan, China) were used. The protein bands were visualized using a Pierce ECL Western Blotting Kit (Pierce, Rockford, IL, USA). All target proteins were normalized to GAPDH. Signals were detected and analyzed using Tanon-4500 Gel Imaging System (Tanon Science and Technology Co., Ltd., Shanghai, China).

2.4. Stub-RFP-Sens-GFP-LC3 lentivirus infection and confocal microscopy autophagy flux measurement

H9c2 cardiomyoblasts were infected with Stub-RFP-Sen-GFP-LC3 lentivirus (Genechem, Shanghai, China). The stable expression of GFP and RFP double-labeled LC3 cells were seeded onto glass confocal dishes. The positive control group was deprived of culture medium for 4 h prior to imaging and treated with EBSS for starvation-induced autophagy. Forty-eight hours after transfection with miR-208a-3p mimics, siPDCD4, and negative control (NC), the cells were visualized under a confocal fluorescence microscope (**Leica**, Wetzlar, Germany).

2.5. Dual-luciferase reporter assay

The total length of the 3′-UTR of PDCD4 was cloned into the PmirGLO Dual-luciferase vector (Promega, Madison, WI, USA) to generate a PmirGLO-PDCD4 wild-type plasmid (PDCD4-WT) (Genwize, Suzhou, China). To examine the binding specificity, we mutated the wild-type plasmid, changing the sequence from GUCUUAA to CAGA AUU. The mutant plasmid (PDCD4-Mut) was confirmed through sequencing. The wild-type and mutant plasmids were respectively co-transfected into HEK293T cells with a miR-208a-3p mimics or its negative control (miR-NC) using Fugene 6 transfection reagent (Promega, Madison, WI, USA). After transfection for 48 h, luciferase activity was assayed using a luciferase assay kit (Promega, Madison, WI, USA).

2.6. MiRNA mimics, PDCD4 siRNA(siPDCD4), and PDCD4 overexpression plasmid transfection

We transfected the miR-208a-3p mimics (RiboBio, Guangzhou, China) using Fugene 6 transfection reagent in serum-free Opti-MEM medium (Gibco, Grand Island, NY, USA) and then gently added the mixture to the cell medium. SiRNA (sequence: GCGATTCAGTCAGTGATAA) targeting rat PDCD4 mRNA and a negative control were designed by the RiboBio Co. (RibioBio, Guangdong, China). A GV144-PDCD4 overexpression plasmid was purchased from Genechem (Shanghai, China). An empty vector plasmid served as the vector control. The overexpression plasmid or siRNA was transfected into H9c2 cardiomyoblasts using Fugene 6 transfection reagent. Forty-eight hours after transfection, total RNA and protein were isolated.

2.7. Statistical analyses

Data were analyzed using GraphPad Prism software 5 (GraphPad Software, Inc. La Jolla, CA, USA). For comparison between two treatment groups, Student's *t*-test was applied. When more than two groups were compared, one-way analysis of variance (ANOVA) was used followed by Bonferroni's post-test. The results are presented as the means ± standard error of the mean (SEM). A P value < 0.05 was considered statistically significant.

3. Results

3.1. MiR-208a-3p is upregulated during autophagy, and miR-208a-3p overexpression augments BNP and β-MHC expression

First, miR-208a-3p expression was significantly elevated in H9c2 cardiomyoblasts after Ang II treatment based on an RT-PCR analysis (Fig. 1A). We increased miR-208a-3p expression using miR-208a-3p mimics. As shown in Fig. 1A, cells transfected with miR-208a-3p mimics showed an approximately eight-fold increase in miR-208a-3p expression compared to that of the NC group, indicating that the transfection efficiency was high. As shown in Fig. 1B and C, cells stimulated with miR-208a-3p mimics or Ang II showed higher expression levels of *BNP* and *β*-MHC, which are molecular markers of hypertrophy, than those of cells not treated with mimics or Ang II [15]. We induced cell autophagy with EBSS by depriving cells of glucose in DMEM for 0, 2, 4, and 6 h. MiR-208a-3p expression levels increased over time during starvation in EBSS, and at 6 h, a significant increase in miR-208a-3p was observed, which is likely relevant to autophagy (Fig. 1D).

3.2. MiR-208a-3p overexpression increases the LC3BII/I ratio, decreases P62, and induces unimpeded autophagy flux

To evaluate whether miR-208a-3p regulates Ang II-mediated