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MICAL3 AFFECTS MYOCARDIAL HYPERTROPHY BY REGULATING CK2A1/HDAC2 PATHWAY

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Abstract

OBJECTIVE: In order to study the role and mechanism of MICAL3 in Ang II-induced myocardial hypertrophy, with cardiomyocyte H9C2 as the focus, the paper established a model of Ang II-induced myocardial hypertrophic cells, and profoundly explored the role of MICAL3 in myocardial hypertrophy and the molecular mechanism.

Methods: The constructed expression plasmid of MICAL3 gene and the Control plasmid were transfected into H9C2 cells. Ang II stimulation was performed. Immunofluorescence technique was used to detect the degree of hypertrophy of H9C2 cells. Fluorescent probes and microplate reader were employed to detect the level of ROS in cardiomyocytes and the HDAC2 activity. Western blot method was applied to detect the phosphorylation level of HDAC. Immunoprecipitation was used to detect the effect of MICAL3 on the interaction between CK2α1 and HDAC2.

Results: The overexpression of MICAL3 protein can block the hypertrophy of cardiomyocytes caused by Ang II, and significantly increase the level of ROS in cardiomyocytes while reducing HDAC2 activity. MICAL3 can compete with HDAC2 and affect the interaction between HDAC2 and CK2α1, thereby reducing the phosphorylation level of HDAC2 at S394 site.

Conclusion: MICAL3 can negatively regulate the hypertrophy of cardiomyocytes caused by Ang II. On the one hand, MICAL3 can competitively inhibit the interaction between HDAC2 and CK2α1, reduce the phosphorylation level of HDAC2 at S394 site and inhibit the activity of HDAC2. On the other hand, MICAL3 inhibits the activity of HDAC2 through the self-generated ROS, thereby inhibiting the HDAC2 positive regulation of myocardial hypertrophy and inhibiting the hypertrophy of myocardial cells.

Keywords: MICAL3; Myocardial Hypertrophy; Ang II; CK2α1/HDAC2

Studies have shown that the MICAL family plays a vital role in regulating the dynamic changes of the cytoskeleton, and then participates in the developmental processes of Drosophila bristles, dendritic spine construction, axon guidance, hippocampal mossy fiber connection, podocyte morphology, and heart formation [1-3]. In addition to participating in the normal development process, MICAL is also closely related to pathological processes such as tumorigenesis, cerebral ischemia, spinal cord injury, and epilepsy [4-5]. MICAL3 is abundantly expressed in heart tissues and plays an important regulatory role in heart formation. However, whether it will also function in the occurrence and development of myocardial hypertrophy has not been reported yet. This study mainly focused on H9C2, established a model of Ang II-induced myocardial hypertrophic cells, and explored the role and molecular mechanism of MICAL3 in myocardial hypertrophy.

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Methods

1. The construction of msMICAL3 eukaryotic expression plasmid and truncated mutant and cell transfection.

With reference to the mouse MICAL3 protein sequence and the construction of the msMICAL3 eukaryotic expression plasmid and truncated mutant, the primers were designed according to Primer 6.0, as follows:

ms-HA-MICAL3:
5'- GCGAATCGACCATGGATGA AGATGAG-3',
5'-GCGATATCGCCACATGGAGAAGATGAG-3';
ms-HA-MICAL3-FM:
5'-GATATCCAT GGAGAGAGGATGAG-3',
5'-GCGCGCCACCTCACTCGGAAGCGGTC-3';

ms-HA-MICAL2-ΔFM:
5'-ATATCCACCAGAGCTCTGAGGAGA-3',
5'-CGCGCCACCTCAGCCAGAAGT TAG-3'.
Cell transfection was performed according to the instructions. Western blot was applied to detect the expression of genes carried by the transfected plasmid.

2. Cell Grouping

The cells were grouped as follows: A: controll + HA, B: AngII + HA, C: controll + HA-MICAL3, and D: AngII + HA-MICAL3.

3. Detection of ROS levels in myocardial cells

H9C2 cells were seeded in a 6-well plate, added with serum-free DMEM medium containing H2DCF-DA fluorescent probe, then incubated at 37 ℃ in darkness for 30 min, and centrifuged (1000 rpm, 5 min). Fluorescence intensity was measured by flow cytometry, with the excitation wavelength at 488 nm.

4. HDAC2 activity detection

The nucleus components of the treated cells were extracted. After protein quantification, the HDAC2 antibody was incubated overnight, and then 25 μl Protein A beads were added and the cells were incubated at 4 °C for 1 h. Each group was incubated with HDAC Assay Buffer and HDAC Substrate and developer. The kit explains the operation procedure. The microplate reader reads the fluorescence value, the excitation light is 380 nm, and the emission light is 550 nm.

5. Co-immunoprecipitation detection

Each group of cells were added with cell lysate. Protein A agarose was added to the cell suspension and incubated to remove non-specific contaminants. The supernatant was added with the corresponding antibody. Protein A agarose was added to capture the antigen-antibody complex. The agarose beads-antigen-antibody complex was collected. The supernatant was discarded. The agarose beads-antigen-antibody complex was suspended with SDS-Page electrophoresis loading buffer. The cells were mixed gently. The sample was heated. The supernatant was detected by Western Blot.

6. Statistical analysis

All data were analyzed using SPSS17.0 software package. Measurement data were expressed as mean ± standard deviation (x ± SD). Comparison between the two groups was performed by independent sample test. $P<0.05$ is considered a statistically significant difference.

Results

1. Overexpression of MICAL3 protein blocked cardiomyocyte hypertrophy caused by Ang II

In order to explore the role of MICAL3 protein in myocardial hypertrophy, HA-MICAL3 expression plasmid and control plasmid were firstly transfected into H9C2 cells respectively. After stimulation with saline and Ang II for 48 h, the surface area of myocardial cells was measured by fluorescent staining technique to explore the role of MICAL3 gene in hypertrophic cardiomyocytes. The results showed that, compared with group A, the surface area of cardiomyocytes increased significantly in group B stimulated by Ang II. While the group D, which was stimulated by Ang II under the same conditions and transfected with HA-MICAL3 plasmid, had a significant decrease in cell surface area. There was no significant difference in group C cells transfected with HA-MICAL3 plasmid but not stimulated by Ang II, suggesting that MICAL3 protein can block cardiomyocyte hypertrophy caused by Ang II, as shown in Figure 1.

2. Overexpression of MICAL3 protein increased ROS levels in cardiomyocytes

The results found that, compared with group A, the intracellular ROS level significantly increased in group B. Compared with group A, when cardiomyocytes were transfected with HA-MICAL3 plasmid, intracellular ROS levels also significantly increased in group C. However, there was no significant increase in intracellular ROS levels in group D. The results further verified that MICAL3 can increase the ROS level in cardiomyocytes, as shown in Figure 2.

3. MICAL3 relied on ROS to inhibit HDAC2 activity

The results showed that: compared with group A, the activity of HDAC2 in H9C2 cells significantly increased in group AB. When HA-MICAL3 protein was overexpressed, the activity of HDAC2 was significantly reduced, suggesting that the overexpression of HA-MICAL3 protein can block the increase of HDAC2 activity caused by Ang II stimulation. And, the higher the level of HA-MICAL3 expressed by the cell, the lower the level of HDAC2. Since the various functions of the MICAL family are realized through the H_2O_2 produced by its own flavin monooxygenase activity, in the experiment, CCG1423 was used to explore whether MICAL3's inhibition of HDAC2 activity depends on its own flavin mono-oxidase activity. The results showed that, the effect of HA-MICAL3 overexpression on reducing HDAC2 activity was obviously inhibited by CCG1423, as shown in Figure 3.

4. MICAL3 inhibited the phosphorylation level of HDAC2 protein S394 site by competing with CK2α1

The results showed that, Ang II stimulation significantly increased the phosphorylation level of HDAC2 S394 site in H9C2 cells. When HA-MICAL3 protein was expressed, the phosphorylation level of this site was significantly reduced, suggesting that the overexpression of HA-MICAL3 can downplay the role of Ang II in increasing HDAC2 S394 phosphorylation level. The immunoprecipitation experiment showed that: when Ang II stimulated H9C2 cells, the interaction between CK2α1 and HDAC2 was enhanced, while when HA-MICAL3 protein was expressed, the enhancing effect was significantly reduced. As the expression level of HA-MICAL3 was further increased, the interaction between CK2α1 and HDAC2 was further suppressed, see Figure 4.

Discussion

Under the stimulation of pressure, volume load, or neurohumoral factors, myocardial hypertrophy, an adaptive compensatory response of the heart, occurs. Continuous myocardial hypertrophy will eventually lead to a variety of serious consequences such as heart failure, or even cardiac death. Studies have confirmed that various cardiac hypertrophy stimulation signals can activate intracellular signal transduction pathways, thereby participating in the occurrence and development of cardiac hypertrophy [6-7]. In-depth interpretation of the signal transduction pathways involved in cardiac hypertrophy will help clarify the molecular mechanism of cardiac hypertrophy, and simultaneously provide new ideas for the development of effective intervention targets.

Cardiovascular mortality still ranks first among all diseases, even higher than tumors. As the number of people living with the disease increases and the population is aging, the world must recognize the seriousness and solve the prevention and treatment problem of cardiovascular disease. Myocardial hypertrophy is the structural and functional changes of the heart in a variety of CVD states such as hypertension. It is manifested as the increase in the volume of myocardial cells, sarcomere hyperplasia, and the reactivation of fetal genes related to myocardial hypertrophy. In the early stage of the pathological process of CVD, myocardial hypertrophy is an adaptive response of the heart, which helps to improve heart function and lower ventricular wall tension. However, long-term and persistent pathological myocardial hypertrophy often leads to cardiac dilation, diastolic dysfunction and a series of decompensation response, and even causes heart failure and sudden death [8].

Various myocardial hypertrophy stimulating signals can activate the relevant receptors on the myocardial cell membrane, thereby activating a series of cell signal transduction pathways, inducing the expression of embryonic genes, promoting phenotypic changes in cardiomyocytes, and ultimately leading to cardiomyocyte hypertrophy. Clinically, hypertension is the most common cause of myocardial hypertrophy. The excessive activation of the renin-angiotensin system and the signaling pathway mediated by AngII through receptors are considered to be the important mechanism that causes hypertension and induce myocardial hypertrophy. Therefore, angiotensin converting enzyme inhibitors and angiotensin receptor antagonists are clinically used to block or delay myocardial hypertrophy caused by pressure load [9]. However, recent studies have found that the role of Ang II in myocardial hypertrophy and ventricular remodeling is not so simple. The combined action of Ang II and other factors is the key to myocardial hypertrophy [10]. It is necessary to continue the study on the pathogenesis of myocardial hypertrophy. Only in this way can the intervention targets of myocardial hypertrophy be discovered. The target plays an important role in the prevention and treatment of clinical diseases and the development of new drugs.

The MICAL family was discovered by studying CasL-mediated signaling pathways, and the initial discovery was MICAL1, which can interact with CasL in the thymus cDNA library. Since then, based on the similarity of amino acid sequence and structure, two other members of the MICAL family, MICAL2 and MICAL3, have been identified in humans and rodents. The MICAL family is an evolutionarily highly conserved multifunctional domain protein, including the N-terminal flavoprotein monooxygenase domain (FM), calponin homology (CH) domain, and LIM (Lin11, Isl-1, and Mec-3) functional domain and carboxyl terminal region [11-12].

HDAC2 plays a role in promoting myocardial hypertrophy, but the expression level of HDAC2 does not change significantly, and is mainly manifested as an increase in activity, which is mainly regulated by post-translational modifications, such as phosphorylation and acetylation. The study found that the phosphorylation levels of S394 and S411 of HDAC2 were significantly increased in the hypertrophic mouse myocardial tissue. When the S394, S422 and S424 sites were mutated, the activity of HDAC2 was significantly reduced. In the myocardial hypertrophic tissue, the activation of HDAC2 is

mediated through S394 phosphorylation sites. Overexpression of wild-type HDAC2 can significantly induce cardiac hypertrophy, while overexpression of mutant HDAC2 S394A cannot induce cardiac hypertrophy [13-14].

Our research found that, the overexpression of MICAL3 protein can significantly block cardiomyocyte hypertrophy caused by Ang II. In the meantime, the overexpression of MICAL3 protein can increase the level of ROS in cardiomyocytes, thereby inhibiting HDAC2 activity. In addition, the overexpression of MICAL3 protein can compete with HDAC2 to bind to CK2α1, resulting in a decrease in the phosphorylation level of S394 of HDAC2. Ang II can significantly increase the phosphorylation of HDAC2 S394 site, and the overexpression of MICAL3 can block the phosphorylation level of HDAC2 S394 site, which is necessary for the activation of HDAC2. Therefore, the overexpression of MICAL3 can inhibit the activity of HDAC2 and further inhibit myocardial hypertrophy.

In summary, this study explored the mechanism of MCIAL3's involvement in myocardial hypertrophy. MCIAL3 affects the process of myocardial hypertrophy by regulating the CK2α1/HDAC2 pathway. However, whether MICAL3 affects other pathways has not yet been elucidated and needs further research.

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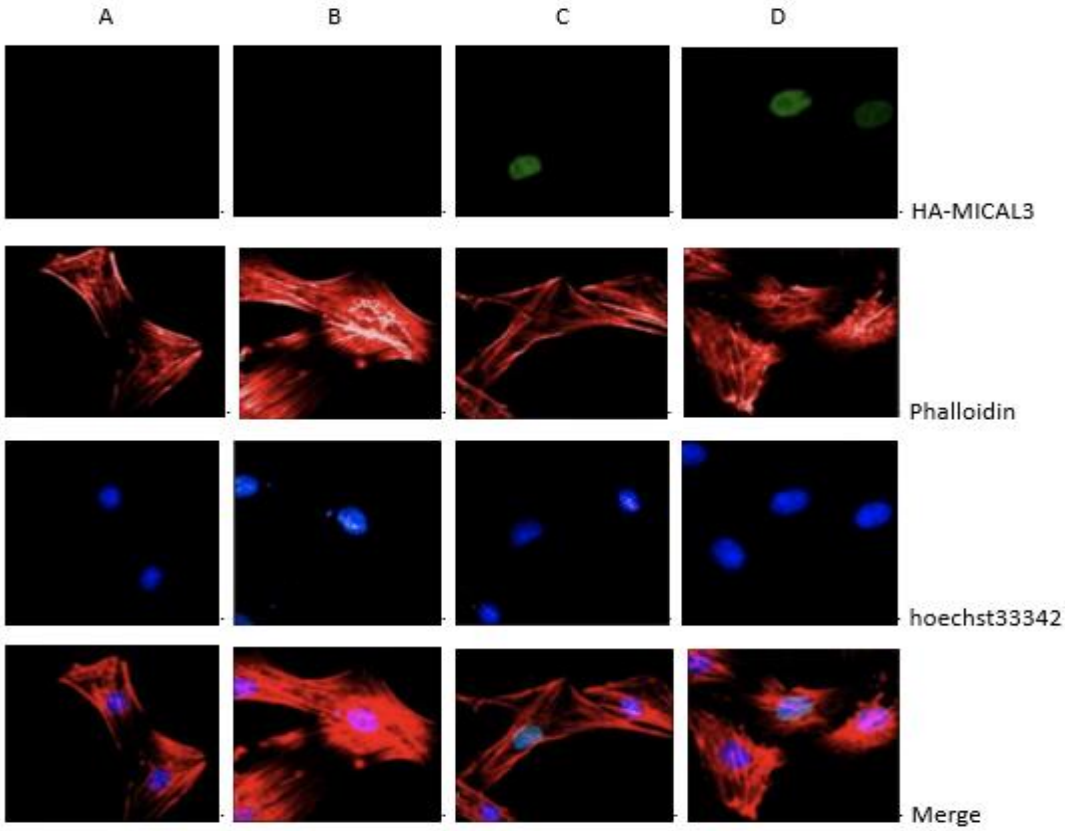

Figure 1. **The results of immunofluorescence staining of rat cardiomyocytes H9C2.**

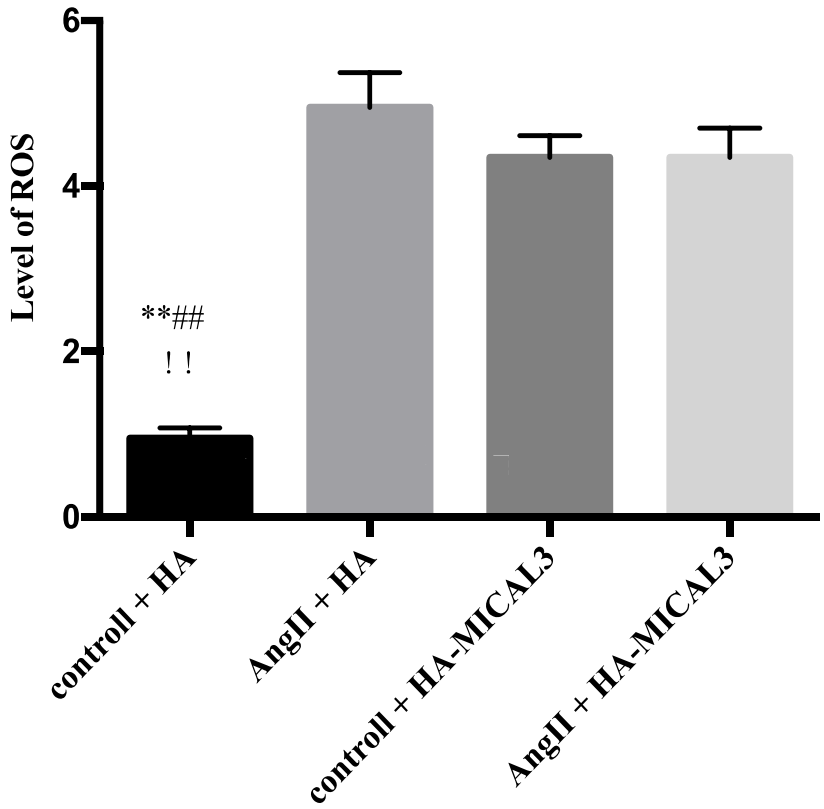

Figure 2. **Overexpression of MICAL2 significantly increased ROS level in cardiomyocytes.**

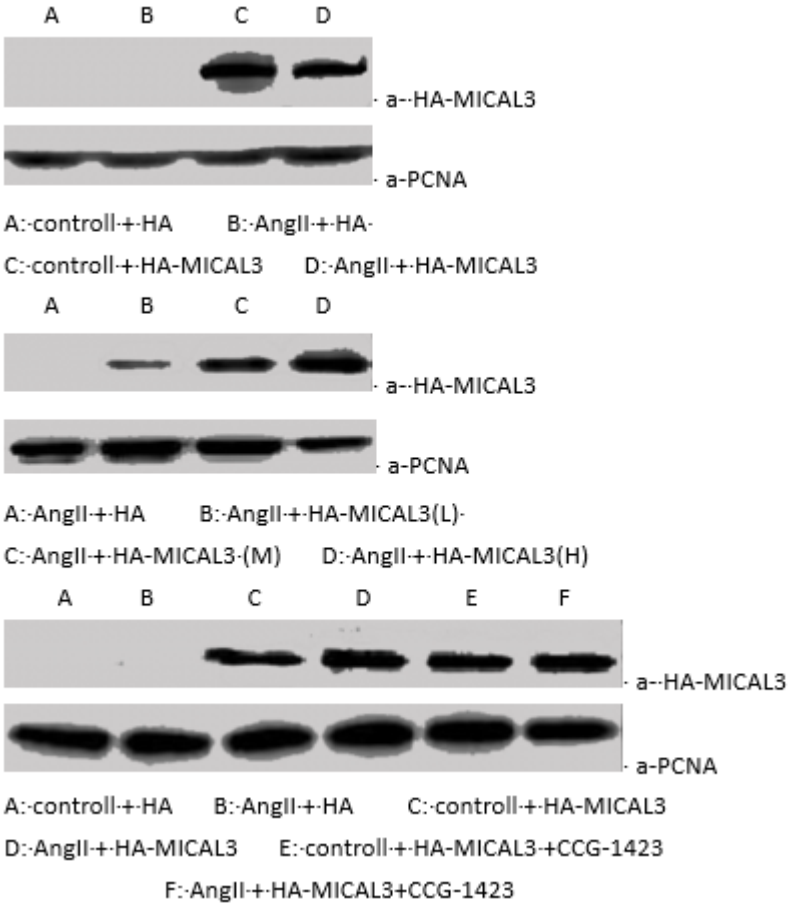

Figure 3. **The result of MICAL3 relying on ROS to inhibit HDAC2 activity.**

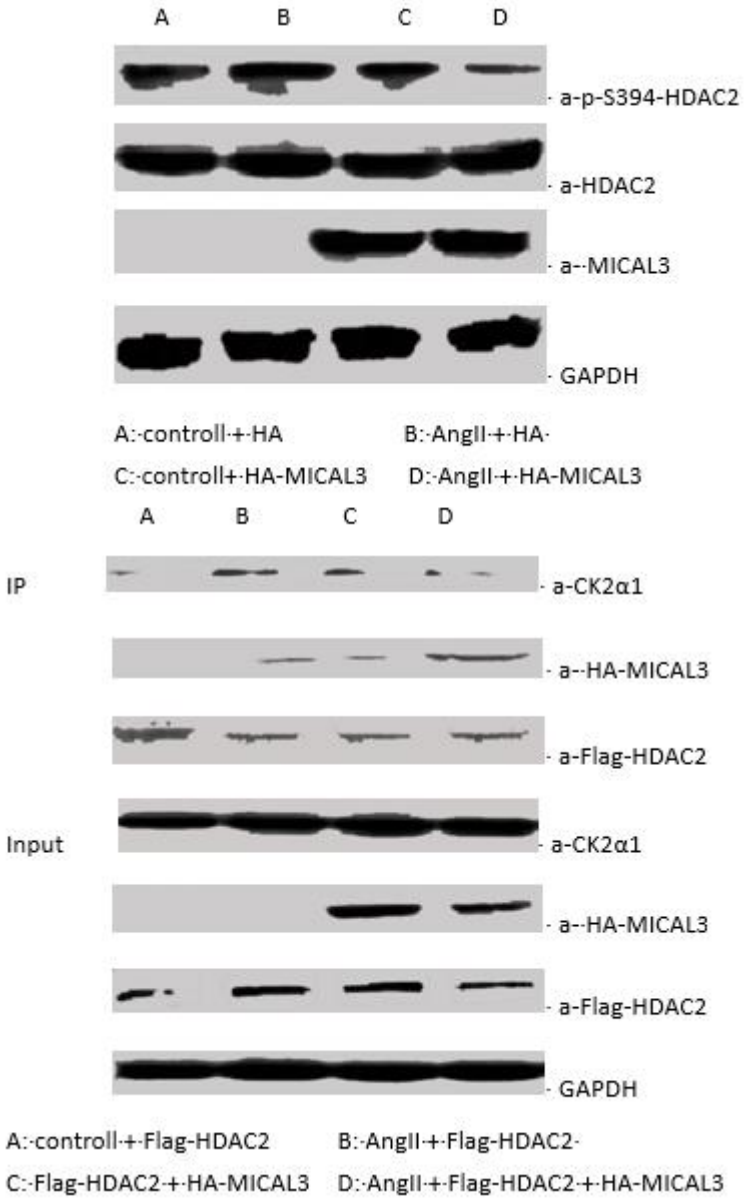

Figure 4. **MICAL3 inhibited HDAC2 protein by competing with CK2α1**