

Odanacatib Inhibits Resistin-induced Hypertrophic H9c2 Cardiomyoblast Cells Through LKB1/AMPK Pathway

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ABSTRACT

Odanacatib (ODN) is a selective inhibitor of cathepsin K. The cysteine protease cathepsin K has been implicated in cardiac hypertrophy. Resistin is an adipokine which is identified to promote cardiac hypertrophy. Here, we hypothesize that ODN mitigates resistin-induced myocyte hypertrophy. Cell surface area and protein synthesis were measured after treatment with resistin and ODN in H9c2 cells. The expression of cardiomyocyte hypertrophy marker BNP and β -MHC was detected by RT-qPCR. The expression and phosphorylation of AMPK and LKB1 were analyzed with Western blot. Resistin could significantly increase cardiomyocyte cell surface area, protein synthesis, and embryonic gene BNP and β -MHC expression, inhibit phosphorylation of AMPK and LKB1. ODN could significantly reverse the effects of resistin. Collectively, our data suggest that ODN can inhibit cardiomyocyte hypertrophy induced by resistin and the underlying mechanism may be involved in LKB1/AMPK pathway.

Key words: Resistin, Odanacatib, LKB1/AMPK pathway, Cardiomyocyte hypertrophy

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INTRODUCTION

Cardiac hypertrophy is compensatory changes of cardiomyocytes caused by all kinds of mechanical stimulation, oxidative stress, neural endocrine hormone and cytokine stimulation. Cardiac hypertrophy is characterized by increased myocardial cell size and protein synthesis, recurrence of fetal genes, change of contraction force and so on. A certain degree of hypertrophy can be compensated, and maintain the normal function of the heart. But continued hypertrophy eventually results in decompensation and lead to change of the structure and function of the heart, and develop to heart failure in the end. Therefore actively control the development of cardiac hypertrophy is important to control the occurrence of heart failure development. It is reported that celecoxib can prevent cardiac hypertrophy and dysfunction by inhibiting apoptosis, inflammation and oxidative stress¹. KMUP-1 attenuates endothelin-1-induced cardiomyocyte hypertrophy through suppression of the Akt/GSK-3 β pathway².

Resistin is a kind of adipokine. It has the effect of insulin resistance. Human resistin gene is located on the 19th chromosome and the molecular weight is 12.5 KDa. Many researchers found that resistin is associated with diseases such as obesity, diabetes and cardiovascular disease. In the myocardial tissue of patients with diabetes, resistin levels are increased significantly³. Resistin can destroy glucose metabolism in myocardial cells, which affects the structure and function of the heart⁴. Recombinant human resistin can aggravate myocardial ischemia-reperfusion injury and increase expression of ANP and BNP via NF- κ B signaling pathway⁵. Resistin is mainly expressed in infarction lesions in animal models of myocardial infarction⁶. Resistin can reduce human coronary artery endothelial nitric oxide synthase expression⁷, increase its permeability⁸ and stimulate monocytes infiltration⁹. Excessive expression of resistin not only promotes myocardial tissue fibrosis, cell apoptosis and oxidative stress but also reduces the contraction force and changes structure of the heart¹⁰.

Cathepsins belong to lysosomal proteases and play important roles in cardiovascular diseases¹¹. Cathepsin K (Cat K) is one of the most important enzymes in the family of lysosomal proteases. Cathepsin K was found in inflammatory macrophages first, it is the key of bone resorption protease^{12, 13}. The expression of cathepsin K is obviously higher in the myocardial tissue of patients with heart failure, dilated and hypertrophic cardiomyopathy^{14, 15}. The expression of cathepsin K is closely related to the development of chronic heart failure, and cathepsin K can be used as a biomarker of cardiac remodeling and dysfunction¹⁶. ODN is a selective and reversible *inhibitor* of cathepsin K. A large number of clinical and experimental studies have found that ODN treatment for osteoporosis has obvious effects¹⁷. ODN can interfere with a variety of precursor and mature cathepsin K transport pathways. The aim of the this study was to evaluate the effects of ODN on resistin-induced cardiomyocyte hypertrophy.

MATERIALS AND METHODS

Reagents

Odanacatib was ordered from Selleckchem (Houston, TX, USA). Recombinant human resistin was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). H9c2 rat cardiomyoblast cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Fetal bovine serum (FBS) was purchased from Zhejiang Tianhang Biological Technology (Zhejiang, China). Antibodies raised against phosphor-LKB1, LKB1, phosphor-AMPK and AMPK were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The UNIQ-10 column TRIzol®

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kit was ordered from Sangon Biotech Co., Ltd. (Shanghai, China). PrimeScript®RT Master Mix Perfect Real Time and SYBR® Premix Ex Taq™ II were obtained from Takara (Tokyo, Japan).

H9c2 cell culture

H9c2 rat cardiomyoblast cells were cultured in DMEM containing 10% FBS, 1% penicillin and 1% penicillin/streptomycin at a temperature of 37°C with a 5% CO₂ atmosphere. Once cells had covered the area of culture bottle bottom 70% to 80%, they were passaged according to a 1:2 or 1:3 proportion. The medium was changed every 2 days. Cells were seeded into a 3.5 cm² culture dish at a density of 1x10⁵. Cells were cultured overnight, and then replaced the medium with serum-free medium for 24 h. Cells were treated with resistin at a concentration of 50 ng/ml¹⁸ and ODN at a concentration of 300 nM¹⁹ for the indicated time.

Determination of cell surface area

8x10⁴ cells were seeded into 3.5 cm² dishes. Cells were cultured with serum-free DMEM for 24 h. Cells were treated with resistin for 48 h or treated with ODN for 2 h and then replaced it with resistin for 46 h. The cell surface area was measured with ImageJ software (NIH ImageJ version 1.49 software, Bethesda, MD, USA). Five observation fields were selected at random and 10 cells in each observation field were selected for measurement of cell surface area¹⁸.

Protein synthesis measurement

1x10⁵ cells were seeded into 3.5 cm² dishes. After cells were cultured with serum-free DMEM for 24 h, cells were treated with resistin for 48 h or treated with ODN for 2 h and then replaced it with resistin for 46 h. The cells were collected and lysed in 70 µl of total protein lysis buffer. Protein concentrations were detected by BCA protein quantitative kit (Boster, Wuhan, China). Cell protein synthesis was determined by dividing the total amount of protein by the cell number¹⁸.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RNA was extracted using the UNIQ-10 column TRIzol kit (Shanghai Sangon Biotech, Shanghai, China) and treated with DNase. A total of 1 µg of RNA was reverse transcribed to cDNA using the PrimeScript®RT Master Mix Perfect Real-Time Kit (Takara). According to the manufacturer's instructions, PCR amplification was conducted with SYBR® Premix Ex Taq™ II kit (Takara) using the Applied Biosystems® 7500 Fast Real-Time PCR System (Thermo Fisher Scientific Inc., Waltham, MA, USA). The PCR reaction condition was: 95°C for 10 min, 95°C for 15 sec, and then 40 cycles of 60°C for 1 min. GAPDH was used as a reference gene. The C_q method was used for relative quantification. The BNP, β-MHC and GAPDH primers were designed and synthesized by Shanghai Sangon Biotech Co., Ltd. The nucleotide sequences of the primers were as follows: BNP forward, 5'-TCTGGGACCACCTCTCAAGT-3' and reverse, 5'-GGCAAGTTTGTGCTGGAAGA-3'; β-MHC forward, 5'-CAGAACACCAGCCTCATCAA-3' and reverse, 5'-CCTCTGCGTTCCTACTCC-3'; GAPDH forward, 5'-ATCACCATCTTCCAGGAGCGA-3' and reverse, 5'-AGCCTTCTCCATGGTGGTGAA-3'.

Western blot analysis

Cells were collected and lysed to prepare protein. The protein concentration was measured with BCA kit (Beyotime Biotechnology, Beijing, China). 5X SDS-PAGE

protein loading buffer (Boster, Wuhan, China) was added to samples, which were heated at 100°C for 5 min. The proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with TBST buffer (20 mM Tris-HCl, 150 mM NaCl and 0.1% Tween-20) containing 5% non-fat milk for 1 h at room temperature. The membranes were incubated in TBST buffer containing 5% non-fat milk with the primary antibodies p-LKB1 (cat. no. 3482S; monoclonal rabbit anti-rat; 1:1,000; Cell Signaling Technology, Inc.), LKB1 (cat. no. 3047S; monoclonal rabbit anti-rat; 1:1,000; Cell Signaling Technology, Inc.), p-AMPK (cat. no. 2531S; polyclonal rabbit anti-rat; 1:1,000; Cell Signaling Technology, Inc.), AMPK (cat. no. 5831S; monoclonal rabbit anti-rat; 1:1,000; Cell Signaling Technology, Inc.), β -actin (cat. no. 4967S; polyclonal rabbit anti-rat; 1:1,000; Cell Signaling Technology, Inc.) at 4°C overnight. After primary antibodies incubation, the membranes were incubated with anti-rabbit secondary antibodies (cat. no. 111-035-003; polyclonal goat-anti rabbit; 1:10,000; Jackson ImmunoResearch, Inc., West Grove, PA, USA) conjugated to horseradish peroxidase at room temperature for 1 h. The protein bands were visualised using a chemiluminescence kit (Boster, Wuhan, China) and FluorChem™Q Quantitative Western Blot Imaging System (Bio-Techne, Minneapolis, MN, USA). The ratio of phosphorylated/unphosphorylated protein was measured with ImageJ software.

Statistical analysis

SPSS 17.0 statistical software was used for data analysis. All experiments data were expressed as means \pm SD. The comparison between multiple groups which satisfied with the normality and homogeneity of variance, the single factor analysis of variance was used, and pairwise comparison with LSD-t method. $p < 0.05$ is statistically different.

RESULTS

ODN treatment decreases H9c2 cell surface area which is increased by resistin

H9c2 cells were treated with resistin at 50 ng/ml for 48 h. Resistin increased cell surface area significantly compared to the control group ($p < 0.01$, Fig. 1). H9c2 cells were pretreated with ODN at 300 nM for 2 h and then treated with resistin. ODN significantly decreased cell surface area compared to the resistin group ($p < 0.01$, Fig. 1).

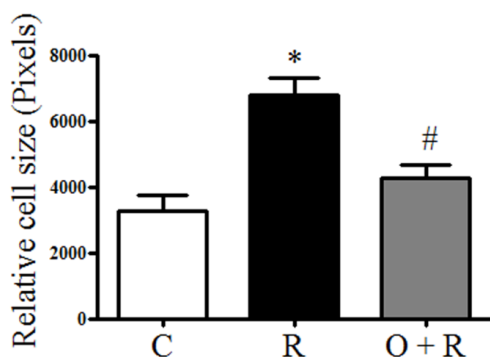


Figure 1 - Effects of ODN on cell surface area increased by resistin. Cell surface areas were measured using ImageJ software. Data represent the mean \pm SD. * $p < 0.01$ vs. the control group, # $p < 0.01$ vs. the resistin group. C, control; R, resistin; O + R, resistin + odanacatib.

ODN opposes the effect of resistin that increases cardiomyocyte protein synthesis

To investigate whether ODN treatment decreases resistin-induced protein synthesis in H9c2 cells, H9c2 cells were treated with resistin at 50 ng/ml for 48 h. Resistin increased protein synthesis significantly compared to the control group ($p < 0.01$, Fig. 2). H9c2 cells were pretreated with ODN at 300 nM for 2 h and then treated with resistin. ODN significantly decreased protein synthesis that was increased by resistin ($p < 0.01$, Fig. 2).

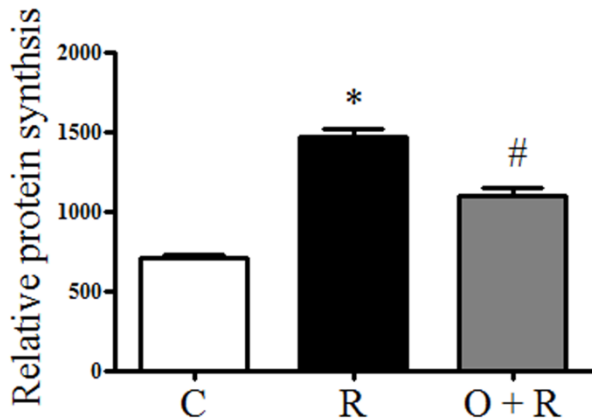


Figure 2 - Effects of ODN on cell protein synthesis enhanced by resistin. Cell protein concentration were measured and normalized to the cell number. Data represent the mean \pm SD. * $p < 0.01$ vs. the control group, # $p < 0.01$ vs. the resistin group. C, control; R, resistin; O + R, resistin + odanacatib.

ODN treatment suppresses the mRNA expression levels of BNP and β -MHC increased by resistin

BNP and β -MHC are markers of cardiomyocyte hypertrophy. The effects of ODN treatment on the expression of BNP and β -MHC mRNA were investigated in H9c2 cells. The results demonstrated that ODN treatment suppressed the expression of BNP and β -MHC mRNA induced by resistin ($p < 0.01$, Fig. 3A, B).

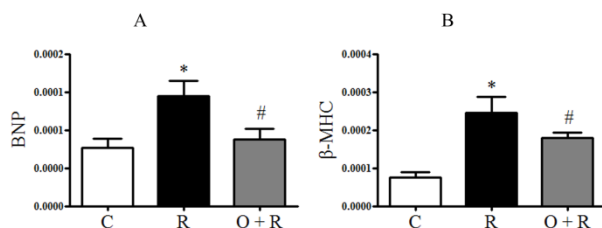


Figure 3 - ODN decreases BNP and β -MHC mRNA expression that is increased by resistin. (A) BNP mRNA and (B) β -MHC mRNA levels were examined by reverse transcription quantitative PCR. Data represent the mean \pm SD. * $p < 0.01$ vs. the control group, # $p < 0.01$ vs. the resistin group. C, control; R, resistin; O + R, resistin + odanacatib.

ODN treatment increases the phosphorylation of LKB1 and AMPK decreased by resistin

To further investigate the underlying molecular mechanism by which ODN inhibits resistin-induced cardiomyocyte hypertrophy, we evaluated the phosphorylation status of LKB1 and AMPK following resistin and ODN treatment by western blot. Treatment of resistin decreased phosphorylation of LKB1 and AMPK, whereas total LKB1 and AMPK protein expression was unchanged. Treatment of ODN increased expression of phosphorylated LKB1 and AMPK that was decreased by resistin (Fig. 4).

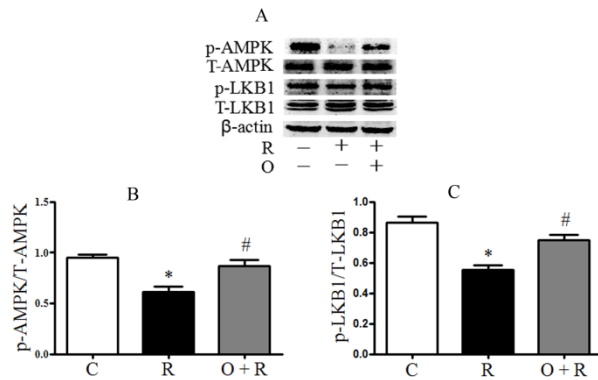


Figure 4 - ODN reverses the effects of resistin that decreases phosphorylated LKB1 and AMPK expression. (A) Representative western blot. (B) The expression of p-AMPK normalized with AMPK. (C) The expression of p-LKB1 normalized with LKB1. Data represent the mean \pm SD. * $p < 0.01$ vs. the control group, # $p < 0.01$ vs. the resistin group. C, control; R, resistin; O + R, resistin + odanacatib.

DISCUSSION

Many cardiovascular diseases such as coronary heart disease, hypertension, heart valve disease, are accompanied by varying degrees of cardiac hypertrophy. Finally, they develop into a heart failure. This is a serious threat to the lives of patients. Therefore, it is great significant to study the mechanism of cardiac hypertrophy for the diagnosis, treatment and prognosis of cardiac hypertrophy and heart failure.

Resistin is a kind of adipokine. In recent years, more and more researches about resistin and cardiac hypertrophy have been studied. Excessive expression of resistin is associated with cardiac remodeling and dysfunction in rat¹⁰. Serum resistin levels are higher significantly in patients with hypertrophic cardiomyopathy²⁰. Resistin overexpression can lead to the increase of the volume of myocardial cells, protein synthesis, the expression of hypertrophy marker ANP, BNP and β -MHC, which are through AMPK/mTOR and JNK/IRS1 signaling pathways²¹. Resistin can also induce myocardial hypertrophy through activating IRS1/MAPK signaling pathway²². In our present study, resistin treatment could increase cell surface area, protein synthesis, expression of BNP and β -MHC, which were consistent with the results of previous studies.

LKB1 is a serine/threonine protein kinase that is expressed in almost all human tissues. LKB1 is a tumor suppressor gene closely related to the occurrence of many kinds of tumors²³. The activation of LKB1 requires the binding of MO25 and STRAD to the complex²⁴. The main function of LKB1 is to inhibit cell proliferation and promote cell apoptosis by inhibiting cell cycle in the G1 phase. LKB1 can phosphorylate downstream AMPK at threonine 172 to activate AMPK. LKB1 is discovered to be the key upstream activator of the AMPK²⁵.

AMPK is a serine/threonine protein kinase, which is widely existed in eukaryotes. It is formed by the three subunit of α , β and γ ²⁶. The activation of AMPK is mainly regulated by the intracellular AMP/ATP ratio, which is an intracellular energy regulator²⁷. Any factors that lead to the increase of AMP/ATP ratio, such as metabolism, oxidative stress, ischemia and hypoxia, can activate the phosphorylation of AMPK. Many studies show that AMPK is closely related to cardiac hypertrophy. The activity of AMPK is significantly decreased in hypertrophic cardiac myocytes, and AMPK can limit the protein synthesis and the remodeling of the cytoskeleton, which can inhibit cardiac hypertrophy²⁸. EGCG prevents phenylephrine-induced myocardial hypertrophy through activating AMPK signaling pathway²⁹. MicroRNA-451 promotes high fat diet induced myocardial hypertrophy by inhibiting the LKB1/AMPK signaling pathway³⁰.

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Cathepsin K is a lysosomal protease involved in various biological activities. The expression of cathepsin K in myocardium of rats with heart failure is significantly higher than that in normal rats. Cathepsin K deficiency can effectively relieve pressure overload cardiac hypertrophy³¹. Cathepsin K induces cardiac hypertrophy by activating the mTOR signaling pathway. Cathepsin K gene knockout can inhibit the hypertrophy and apoptosis of cardiomyocytes, but does not change the myocardial fibrosis³². ODN is an inhibitor of cathepsin K. Through the experiments using ODN to intervene resistin-induced cardiomyocyte hypertrophy, we found that cell surface area, protein synthesis, BNP and β -MHC expression were significantly decreased, while LKB1 and AMPK phosphorylation levels were significantly increased after treatment with ODN and resistin.

CONCLUSION

Resistin can increase cardiomyocyte size, protein synthesis and hypertrophic marker BNP and β -MHC mRNA expression. Resistin also decreases the activation of LKB1/AMPK pathway. Whereas ODN reverses these effects of resistin, suggesting ODN can inhibit resistin-induced H9c2 cell hypertrophy through the activation of LKB1/AMPK signaling pathway.

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