International Journal of Pharmacology

ISSN 1811-7775

science alert

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Asian Network for Scientific Information

OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2017.212.217

Research Article
Odanacatib Inhibits Resistin-induced Cardiomyocyte Hypertrophy Through the Inactivation of ERK Signaling Pathway

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Abstract

Background and Objective: Evidences from human and animal studies have documented elevated levels of lysosomal cysteine protease cathepsin K in failing hearts. Many studies have shown that cathepsin K can cause cardiomyocyte hypertrophy. Odanacatib (ODN) is a kind of non-lysosomes, specificity of cathepsin K inhibitor. Here, it is hypothesized that ODN mitigates resistin-induced cardiomyocyte hypertrophy. The effects of ODN on resistin-induced cardiomyocyte hypertrophy through the extracellular signal-regulated kinases (ERK) signaling pathway were investigated. **Materials and Methods:** Rat cardiomyoblast cells (H9c2) were treated with ODN alone or in combination with resistin. Cell surface area was measured with ImageJ software and protein synthesis was determined by dividing the total protein content by the cell number. Brain Natriuretic Peptide (BNP) and β-myosin heavy chain (β-MHC) were quantitated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Finally, phosphorylation of ERK1/2 was analyzed by Western blot. **Results:** Treatment of H9c2 cells with resistin increased cell surface area, protein synthesis and expression of hypertrophic marker BNP and β-MHC. Pretreatment with ODN attenuated these effects of resistin. Furthermore, treatment with resistin increased phosphorylation of ERK1/2, whereas pretreatment with ODN decreased phosphorylation of ERK1/2 that was increased by resistin. **Conclusion:** The data suggest that ODN can inhibit cardiomyocyte hypertrophy induced by resistin. The underly mechanism may be involved in the inactivation of ERK cell signaling pathway.

Key words: Resistin, odanacatib, cathepsin K, ERK signaling pathway, cardiomyocyte hypertrophy

Received: October 08, 2016 Accepted: October 25, 2016 Published: January 15, 2017

Citation: Xian Zheng, Guanchang Cheng, Jianwei Luo, Qunhui Ye, Yongzhi Deng and Lin Wu, 2017. Odanacatib inhibits resistin-induced cardiomyocyte hypertrophy through the inactivation of ERK signaling pathway. Int. J. Pharmacol., 13: 212-217.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cardiac hypertrophy is an independent risk factor for cardiac morbidity and mortality. The main characteristics of cardiac hypertrophy are increased myocardial cell size and protein synthesis, recurrence of fetal genes and change of contraction force. Despite its initial compensatory is good for pressure overload, persistent cardiac hypertrophy results in heart failure. In recent years, researches of development and treatment of cardiac hypertrophy show that berberine improves pressure overload-induced cardiac hypertrophy through enhanced autophagy[1]. Puerarin suppresses angiotensin II-induced cardiac hypertrophy by inhibiting NADPH oxidase activation[2].

Resistin is an adipokine secreted by human macrophages and rodent animal adipose tissue[3]. Resistin is a 12.5 kDa peptide which its concentration in circulating plasma is 2.5-21.5 ng mL^{-1}. Its receptor has not been identified. But the recent report displays that adenylate cyclase-associated protein 1 (CAP1) may be the receptor of resistin[4]. Overexpression of resistin is related to insulin resistance and dyslipidemia[5]. Expression of resistin is increased in type 1 and type 2 diabetes, in particular, more expression in type 2 diabetes, suggesting that it is closely related to type 2 diabetes. The serum resistin levels are closely related with cardiovascular diseases such as atherosclerosis, coronary heart disease and heart failure[6].

Cathepsins are a group of lysosomal cysteine proteases belonging to the papain family. Accumulating evidence has implicated a role of cathepsins in the pathogenesis of cardiovascular disease. Among the cathepsins, cathepsin K possesses the most potent collagenolytic and elastolytic activities. Cathepsin K is found in inflammatory macrophages and plays a key role in the bone resorption[7]. The expression of cathepsin K is obviously higher in the myocardial tissue of patients with heart failure, dilated and hypertrophic cardiomyopathy[8,9]. Fujita *et al.*[10] found that the serum levels of cathepsin K are significantly increased in patients with atrial fibrillation, which are related to the atrial diameter and extracellular matrix protein peptide[10]. Cathepsin K is mainly found in vascular smooth muscle cells, endothelial cells and inflammatory macrophages in atherosclerosis[11,12]. Cathepsin K levels are significantly increased in the humans and animals who have vascular endothelial injury and atherosclerosis[13]. Inhibition of cathepsin K expression can promote cardiovascular repair and prevent atherosclerotic plaque progression[12]. Some studies have proven that the expression of cathepsin K is closely related to the development of chronic heart failure[14]. Odanacatib (ODN) is an inhibitor of cathepsin K. A large number of clinical and experimental studies have found that ODN treatment for osteoporosis has obvious effects[15]. The ODN can interfere with a variety of precursor and mature cathepsin K transport pathways.

The aim of the present study was to investigate the effects of ODN on resistin-induced cardiomyocyte hypertrophy.

MATERIALS AND METHODS

Reagents: Odanacatib was purchased from Selleckchen (Houston, TX, USA). Recombinant human resistin was ordered from PeproTech, Inc. (Rocky Hill, NJ, USA). The H9c2 rat cardiomyoblast cells were purchased from American Type Culture Collection (Manassas, VA, USA). Fetal bovine serum (FBS) was obtained from Zhejiang Tianhang Biological Technology (Huzhou, Zhejiang, China). The antibodies for phospho-ERK and ERK were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The antibodies for β-actin and goat anti-rabbit IgG were purchased from Bioworld Technology, Inc. (Wuhan, Hubei, China). The UNIQ-10 column TRIzol® kit was obtained from Shanghai Sangon Biotech Co. Ltd. (Shanghai, China). PrimeScript® RT Master Mix Perfect Real Time and SYBR® Premix Ex Taq™ II were ordered from Takara (Tokyo, Japan).

H9c2 cell culture: Cells were cultured in DMEM medium containing 10% fetal bovine serum, 100 U mL^{-1} penicillin and streptomycin at 37°C in a humidified 5% CO_2 atmosphere. The medium was changed every 2 days. Cells were passaged according to a 1:2 or 1:3 proportion when they grew to 70-80% of the dish. Cells were seeded in 6-well plates at densities of 1×10^5 cells $well^{-1}$. Cells were cultured in DMEM without serum for 24 h and then treated with resistin at indicated time.

Determination of cell surface area: The morphology of cells was observed under inverted microscope. Five different fields of the view were randomly selected from each group. 10 of cells were randomly selected from each field of the view and the surface areas of the cells were analyzed by ImageJ software (NIH, Bethesda, MD, USA)[16].

Protein synthesis measurement: Cells were harvested and lysed in 70 μL of RIPA buffer containing 1% PMSF (Beyotime, Beijing, China). Protein concentrations were measured using a BCA Protein Assay Kit (Boster, Wuhan, Hubei, China). Cell protein synthesis was determined by dividing the total amount of protein by the cell number[16].

RT-qPCR: Total RNA was extracted from the cells using the UNIQ-10 column TRIzol kit (Shanghai Sangon Biotech, Shanghai, China). About 1 μg of RNA was reverse transcribed to cDNA using the PrimeScript® RT Master Mix Perfect Real-Time Kit (Takara, Tokyo, Japan). The 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 conditions of PCR reaction were: 95°C for 10 min, 95 for 15 sec, 40 cycles of 60°C for 1 min. The GAPDH was used as a reference gene. The ΔΔCt method was used for relative quantification. The BNP, β-MHC and GAPDH primers were synthesized by Shanghai Sangon Biotech Co. Ltd. The nucleotide sequences of the primers were as follow: BNP forward, 5-TCTGGGACCACCTCTCAAGT-3 and reverse, 5-GGCAAGTTTGTGCTGGAAGA-3; β-MHC forward, 5-CAGAACACCAGCCTCATCAA-3 and reverse, 5-CCTCTGCGTTCCTACACTCC-3; GAPDH forward, 5-ATCACCATCTTCCAGGAGCGA-3 and reverse, 5-AGCCTTCTCCATGGTGGTGAA-3.

Western blot analysis: Cells were collected and lysed with RIPA buffer. Protein concentration was measured. Samples were mixed with 5×SDS-PAGE protein loading buffer (Boster, Wuhan, China), boiled and centrifuged. Each sample was applied to a sodium dodecyl sulfate (SDS) polyacrylamide (12%) gel for electrophoresis. The gel was run at 80 V for 30 min and 120 V for 1 h. Then the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA) for 45 min. The membranes were blocked with 5% BSA in TBST buffer (20 mM tris-HCl, 150 mM NaCl and 0.1% tween-20) for 1 h at room temperature. They were then incubated in TBST buffer containing 5% BSA with the respective primary antibodies p-ERK, ERK and β-actin at 4°C overnight. After washing three times in TBST for 5 min, the membranes were incubated with anti-rabbit IgG-peroxidase conjugated at room temperature for 1 h. The protein bands were visualised using an chemiluminescence kit (Boster, Wuhan, China) and FluorChem™Q Quantitative Western Blot Imaging System (Bio-Techne, Minneapolis, MN, USA).

Statistical analysis: For statistical analysis, SPSS 17.0 statistical software was used. All experiments data were expressed as Means±SD. One-way analysis of variance (ANOVA) and LSD-t test were used for analysis of the experimental data, $p<0.05$ is statistically significant.

RESULTS

ODN reduces cell surface area which is increased by resistin: The H9c2 cells were pretreated with ODN[17] at 300 ng mL^{-1} for 2 h and treated with resistin[16] at 50 ng mL^{-1} for 48 h. Compared to the control group, resistin increased cell surface area significantly ($p<0.01$) (Fig. 1). Compared to the resistin group, ODN significantly decreased cell surface area which was increased by resistin ($p<0.01$) (Fig. 1).

ODN reduces cell protein synthesis which is increased by resistin: The H9c2 cells were treated with resistin at 50 ng mL^{-1} for 48 h. Compared with the control group, resistin increased protein synthesis ($p<0.01$) (Fig. 2). The H9c2 cells were pretreated with ODN at 300 ng mL^{-1} for 2 h and then replace it with resistin for 48 h. The ODN could reduce protein synthesis compared to the resistin group ($p<0.01$) (Fig. 2).

ODN inhibits the mRNA expression of BNP and β-MHC: The BNP and β-MHC are markers of cardiac hypertrophy. The H9c2 cells were treated with ODN or in combination with resistin. The expression of BNP and β-MHC mRNA was increased by resistin compared to the control group (Fig. 3). The expression of BNP and β-MHC mRNA was decreased by ODN compared to the resistin group (Fig. 3).

ODN treatment decreases phosphorylation of ERK1/2: To study whether ODN inhibiting cardiomyocyte hypertrophy was involved in ERK1/2 signaling pathway, the levels of phosphorylated ERK1/2 were detected by Western blot.

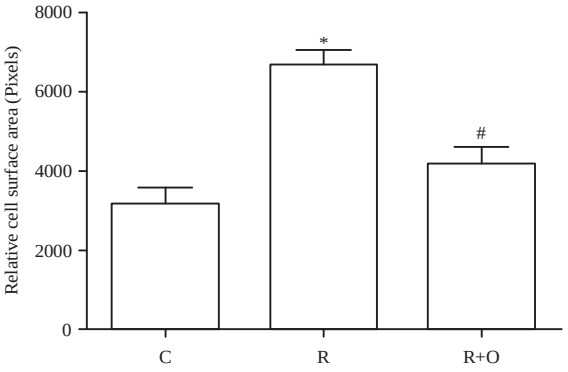

Fig. 1: ODN decreases cell surface area increased by resistin. Data represent Mean±SD, *$p<0.01$ versus the control group, #$p<0.01$ versus the resistin group, C: Control, R: Resistin, R+O: Resistin+odanacatib

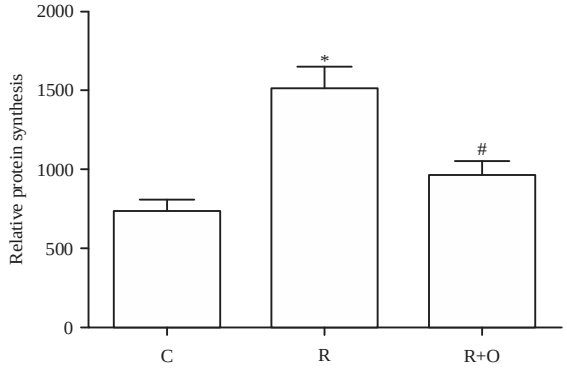

Fig. 2: ODN decreases cell protein synthesis increased by resistin. Data represent Mean±SD, *p<0.01 versus the control group, #p<0.01 versus the resistin group

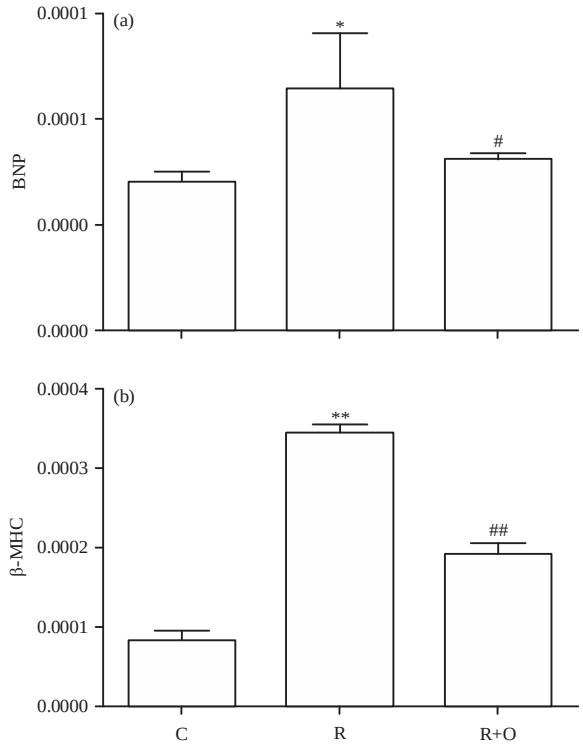

Fig. 3(a-b): ODN reduces BNP and β-MHC mRNA expression increased by resistin, (a) BNP mRNA and (b) β-MHC mRNA levels were examined by reverse transcription quantitative PCR. Data represent Mean±SD, *p<0.05, **p<0.01 versus the control group, #p<0.05 ##p<0.01 versus the resistin group

Treatment of resistin increased phosphorylation of ERK1/2 compared to the control group (p<0.01) (Fig. 4). Pretreated with ODN decreased expression of phosphorylated ERK1/2 that was increased by resistin as compared with the resistin group (p<0.01) (Fig. 4).

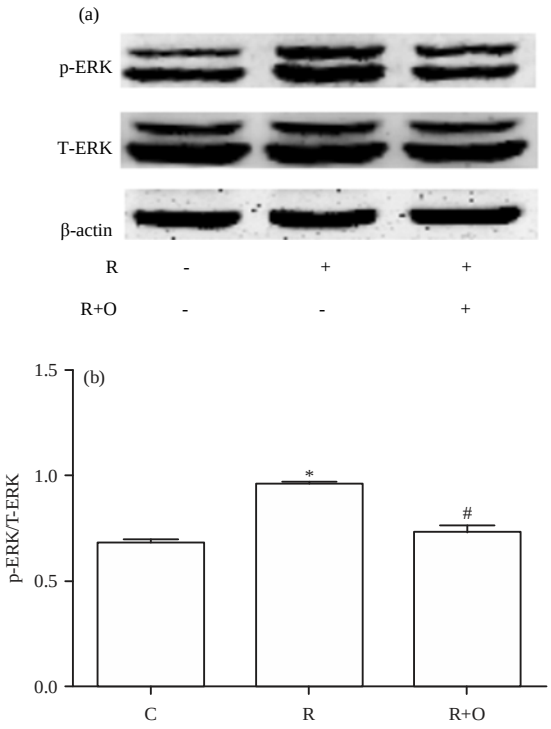

Fig. 4(a-b): ODN decreases phosphorylated ERK1/2 expression increased by resistin, (a) Representive Western blot and (b) The expression of p-ERK1/2 normalized with ERK1/2. Data represent Mean±SD, *p<0.01 versus the control group, #p<0.01 versus the resistin group

DISCUSSION

Many cardiovascular diseases 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 mechanisms of cardiac hypertrophy for the diagnosis, treatment and prognosis of cardiac hypertrophy and heart failure. Recent studies have shown that resistin is related to cardiac hypertrophy. Overexpression of resistin leads to cardiac dysfunction and cardiac remodeling[18]. Resistin worsens cardiac ischemia reperfusion injury[19]. Overexpression of resistin is also associated with activation of the mitogen-activated protein kinases (MAPK) such as ERK1/2 and p38, as well as increased Ser-636 phosphorylation of insulin receptor substrate-1 (IRS-1), indicating that IRS-1/MAPK pathway is involved in cardiac hypertrophy[20]. Resistin can destroy glucose metabolism in myocardial cells, which affects the structure and function of the heart[21]. In these experiments, resistin increased cardiomyocyte size and protein synthesis. At the same time, resistin increased mycardial hypertrophy

marker BNP and β-MHC mRNA expression. It suggests that resistin induces cardiomyocyte hypertrophy, which is consistent with previous studies[18,20].

Numerous studies have demonstrated that MAPK signaling pathway plays a critical role in the pathogenesis of cardiac hypertrophy and heart failure[22]. The MAPK cascade comprises a sequence of successive kinases, including ERK1/2, c-Jun NH_2-terminal kinases (JNK1, JNK2 and JNK3) and p38 kinase[22]. The prototypic ERK pathway is found to be mainly responsive to stimulation by growth factors[23]. Cardiac hypertrophy is related to the activation of ERK signaling pathway. Cinnamaldehyde can retard the progression of cardiac hypertrophy and fibrosis via blocking ERK signaling pathway[24]. Epidermal growth factor receptor (EGFR) inhibitors markedly inhibit both ERK1/2 and AKT activation in response to angiotensin II (Ang II)-induced cardiac hypertrophy[25]. Downstream targets of ERK and PI3K/Akt include transcription factors such as NF-kB, AP-1 and Smad, which have been shown to regulate inflammation and fibrosis respectively[26,27]. Mounting evidence has strongly suggested that inflammation and fibrosis play a key role in the development of cardiac hypertrophy and heart failure[28]. Naringenin attenuates pressure overload-induced cardiac hypertrophy via PI3K/Akt, ERK and JNK signaling pathways[29]. In these studies, resistin increased phosphorylation of ERK1/2. Whereas ODN inhibited phosphorylation of ERK1/2 that was induced by resistin.

Cathepsin K is a lysosomal protease which is involved in many kinds of biological activities. It is closely related to the occurrence and development of cardiac hypertrophy. It is found that expression of cathepsin K in the myocardium of heart failure rats is obviously higher than that in normal rats. Cathepsin K deficiency can effectively relieve pressure overload cardiac hypertrophy[30]. Cathepsin K induces cardiac hypertrophy by activating the mTOR signaling pathway. Cathepsin K knockout mitigates high-fat diet-induced cardiac hypertrophy and contractile dysfunction. Cathepsin K knockout also improves calcium metabolism disorders, relieves the high fat diet-induced glucose intolerance and myocardial insulin resistance and prevents apoptosis of myocardial cells induced by fatty acids stimulating cytochrome C release[31]. Cathepsin K knockout can inhibit hypertrophy and apoptosis of cardiomyocytes, but can't change myocardial fibrosis[32]. Using ODN to intervene resistin-induced cardiac hypertrophy, it is found that resistin increased cell surface area, protein synthesis, BNP and MHC mRNA expression. Resistin also increased phosphorylation of ERK. The ODN significantly reversed these effects of resistin.

CONCLUSION

In conclusion, ODN inhibited cell surface area, protein synthesis, BNP and β-MHC mRNA expression increases that are induced by resistin. Furthermore ODN repressed phosphorylation of ERK that is increased by resistin. It is suggesting that ODN inhibits cardiomyocyte hypertrophy through the inhibition of ERK signaling pathway.

ACKNOWLEDGMENTS

The present study was supported by the Key Research and Development Program of Shanxi Province (Grant No. 201603D321057) and Basic and Cutting-Edge Technology Research Project of Henan Province (Grant No. 142300410118).

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