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Research Article
Inhibitory Effect of Apelin on Cardiomyocyte Hypertrophy induced by Resistin in H9c2 Cells

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Abstract

Background and Objective: Resistin induces cardiac hypertrophy, while apelin inhibits cardiac hypertrophy. But the underlying molecular mechanisms are still not clear that apelin inhibits cardiac hypertrophy induced by resistin. The research purpose was to investigate the underlying molecular mechanism that apelin represses resistin-induced cardiomyocyte hypertrophy. **Materials and Methods:** H9c2 cells were used to measure surface area of cells and protein synthesis. RT-qPCR was performed to analyze hypertrophic marker brain natriuretic peptide (BNP) and β-myosin heavy chain (β-MHC) mRNA expression. Western blot was employed to examine phosphorylation of LKB1 and AMPK. **Results:** The results displayed that cell surface area, protein synthesis, BNP and β-MHC mRNA expressions were increased with resistin treatment. While apelin reversed these effects of resistin and this was further blocked by the electrophilic aldehyde lipid peroxidation byproduct 4-hydroxy-2-nonenal (HNE). Furthermore, resistin decreased phosphorylation of LKB1and AMPK, whereas pre-cultured with apelin increased phosphorylation of LKB1and AMPK that was decreased by resistin, which was blocked by HNE. **Conclusion:** These results suggested that apelin can inhibit cardiomyocyte hypertrophy induced by resistin through the activation of LKB1/AMPK cell signaling pathway.

Key words: Apelin, cardiac hypertrophy, resistin, cardiomyocyte, natriuretic peptide, protein synthesis, LKB1/AMPK signaling pathway

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

INTRODUCTION

Cardiomyocyte hypertrophy is the end-stage performance of various kinds of heart disease, such as hypertension, myocardial infarction and valvular heart disease. The mainly features of cardiac hypertrophy include increased cell size, protein synthesis, embryonic gene atrial natriuretic peptide (ANP), BNP and β-MHC expression and enhanced fibronectin. There are lots of cell signaling pathways related to cardiac hypertrophy and cardiac failure[1,2].

Resistin is linked to obesity, diabetes, insulin resistance and cardiac hypertrophy[3,4]. Resistin impairs cardiac recovery following ischemia[5], stimulates cardiac TNF-alpha secretion and modulates reperfusion release of natriuretic peptides and biochemical markers of myocardial damage[6]. Recombinant resistin is sufficient to re-constitute hepatic insulin resistance. Loss of resistin can improve insulin sensitivity[7]. Resistin can lead to glucose intolerance. Cytokines such as endothelin (ET), insulin, insulin-like growth factors (IGFs) and peroxisome proliferator-activated receptor γ (PPARγ) can regulate resistin secretion[8,9]. Human resistin is primarily expressed in macrophages and neutrophils, while resistin is located to adipocytes in rodents[10,11]. Human resistin is response to inflammatory stimuli, which is found to be elevated in autoimmune disease and sepsis[12].

Apelin is an endogenous peptide ligand for the G-protein coupled apelin receptor (APJ). Apelin and APJ can express in the heart[13]. Apelin is made up of 77 amino acid peptide synthesis, which can process into various C-terminal fragments, including: apelin-36, apelin-19, apelin-17, apelin-13, apelin-12 and [Pyr1]-apelin-13. Apelin-13 is more stable thereinto[14]. Loss of apelin accentuates myocardial infarction (MI) adverse remodeling and injury of ischemia-reperfusion[15]. Treatment of apelin protects the heart against ischemia-reperfusion injury through inhibition of endoplasmic reticulum (ER)-dependent apoptotic pathways in a time-dependent fashion[16]. Apelin-13 increases myocardial progenitor cells and improves repair post myocardial infarction[17]. Apelin also ameliorates high fat diet-induced cardiac hypertrophy and contractile dysfunction[18]. It has been known that resistin induces cardiac hypertrophy and apelin inhibits cardiac hypertrophy, but whether apelin inhibition of cardiomyocyte hypertrophy induced by resistin is not clear. The purpose of this study was to explore the underlying molecular mechanism that apelin inhibits cardiomyocyte hypertrophy induced by resistin through the activation of LKB1/AMPK signaling pathway.

MATERIALS AND METHODS

Reagents: This research project was conducted from January 1, 2015 to July 1, 2017 at the Central Laboratory of Shanxi Cardiovascular Hospital (Taiyuan, Shanxi, China). Apelin-13 was ordered from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). Recombinant human resistin was purchased from Preprotech (Rocky Hill, NJ, USA). H9c2 cells (Rat cardiomyoblast cells) were obtained from the American Type Culture Collection (Manassas, VA, USA). Fetal calf serum was ordered from Zhejiang Tianhang Biological Technology (Zhejiang, China). Phospho-AMPK antibody, Phospho-LKB1 antibody, AMPK antibody and LKB1 antibody were bought from Cell Signaling Technology (Danvers, MA, USA). UNIQ-10 column Trizol Kit was ordered from Shanghai Sangon Biotech (Shanghai, China). PrimeScript®RT Master Mix Perfect Real Time and SYBR® Premix Ex *Taq*™ II were obtained from Takara (Tokyo, Japan).

H9c2 cell culture: The H9c2 cells were cultured in DMEM containing 10% FBS, 1% penicillin and 1% streptomycin at a temperature of 37°C with a 5% CO_2. When H9c2 cells were grown to 80%, cells were passaged according to the proportion of 1:2. The medium was replaced for every 2 days. The experiments were divided four groups: (1) Control. (2) Resistin treatment group. (3) Apelin+Resistin treatment group. (4) HNE+Apelin+Resistin treatment group. Cells were inoculated into 35 mm dishes for 24 h and then cultured overnight in a serum-free medium. Cells with 80% confluence were used for experiments.

Measurement of cell surface area: Cells were inoculated into 35 mm dishes at 8×10^4 for 24 h and then cultured overnight in a serum-free medium. For the resistin group, cells were treated with resistin[19] at 50 ng mL^{-1} for 48 h. For the Apelin+Resistin treatment group, cells were pre-treated with apelin[20] at 100 nmol for 1 h and then treated with resistin at 50 ng mL^{-1} for 48 h. For the HNE+Apelin+Resistin treatment group, cells were treated with HNE[21] at 20 μM for 1 h and then treated with apelin at 100 nmol for 1 h, thereafter cells were treated with resistin at 50 ng mL^{-1} for 48 h. Cell surface area was determined with NIH ImageJ software (NIH ImageJ version 1.49 software, Bethesda, MD, USA). Five observation fields were selected at random and 10 of cells in each observation field were selected for measurement of cell surface areas[19].

Protein synthesis measurement: About 1×10^5 cells were cultured in 35 mm dishes. Cells were cultured in serum-free medium overnight and treated with resistin at 50 ng mL^{-1} for 48 h in the presence or absence of 100 nmol apelin for 1 h and 20 μM HNE for 1 h. Cells were digested with trypsin and counted under microscope with cell count plate. At the same time, cells were collected and lysed into 100 μL of RIPA buffer. Protein concentrations were measured using Bradford protein assay kit (Bio Rad, Hercules, CA, USA). Cell protein synthesis was determined by dividing the total amount of protein by the cell number[19].

Real-time PCR: Cells were collected and RNA was extracted using the UNIQ-10 column Trizol Kit (Shanghai Sangon Biotech, Shanghai, China). About 1 μg of RNA was reverse transcribed into cDNA using the PrimeScript® RT Master Mix Perfect Real Time Kit (TaKara, Japan). The PCR amplification was conducted with SYBR® Premix Ex *Taq*™ II Kit (TaKara, Japan) using Applied Biosystems® 7500 Fast Real-Time PCR System (Applied Biosystems, Foster city, CA ,USA). The reaction condition was: 95°C for 30 sec and 40 cycles at 95°C for 5 sec followed by 60°C for 31 sec. About 18S rRNA gene expression was used as a control. The ΔΔCt method was used for relative quantification. The BNP, β-MHC and 18S primers were designed and synthesized by Shanghai Sangon Biotech (Shanghai, China) as following. BNP forward primer:

- 5′-GGAGCATTGAGTTGGCTCTC-3′, reverse primer
- 5′-CCAGCTCTCCGAAGTGTTTC-3′; β-MHC forward primer
- 5′-CACCCGCGAGTACAACCTTC-3′, reverse primer
- 5-CCCATACCCACCATCACACC-3′; 18S forward primer
- 5′-CACCCGCGAGTACAACCTTC-3′, reverse primer
- 5′-CCCATACCCACCATCACACC-3′

Western blot analysis: When H9c2 cells were grown to 80-90% after treatment, they were washed twice with 1×PBS and digested with 0.05% trypsin for 1 min and centrifuged at 1000 g for 5 min. Cells were added with 100 μL of lysis buffer and put in ice for 20 min. Lysates were centrifuged and protein concentration was measured using BCA assay. About 5×Laemmli sample buffer was added to samples. Lysates were heated at 95°C for 5 min and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred into PVDF membranes (Sigma-Aldrich, St. Louis, MO , 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, LKB1, p-AMPK, AMPK and β-actin at 4°C overnight. After primary antibody incubation, the membranes were incubated with anti-rabbit secondary antibodies linked horseradish peroxidase (HRP) at room temperature for 1 h. The bands were visualized with an enhanced chemiluminescence kit (Beijing ComWin Biotech, Beijing, China) using FluorChem Q System Quantitative Western Blot Imaging (Protein Simple, London, UK). The band intensities were measured with NIH ImageJ software and the ratio of phosphorylated protein antibodies over corresponding total protein antibodies was calculated.

Statistical analysis: All experiment data were expressed as Mean±SD and performed at least three times. All statistical analyses were performed by one-way ANOVA followed by the Bonferroni *post hoc* test. The differences were considered significant if $p<0.05$.

RESULTS

Apelin inhibits cardiomyocyte cell size increase that is induced by resistin: In order to induce cardiomyocyte hypertrophy, H9c2 cells were treated with resistin at 50 ng mL^{-1} for 48 h, resistin increased cell surface area significantly as compared to the control group. Pre-treatment of cardiomyocytes with apelin significantly decreased cell surface area that was increased by resistin. If co-cultured with apelin, resistin and HNE, HNE blocked the inhibitory effect of apelin on resistin-increased cell size (Fig. 1).

Apelin decreases cardiomyocyte protein synthesis that is increased by resistin: To test whether resistin treatment increases protein synthesis in H9c2 cells and the increase is inhibited by apelin, cells were exposed to resistin for 48 h in the presence and absence of apelin or HNE+apelin. Resistin significantly increased protein synthesis in cardiomyocytes. Apelin decreased protein synthesis that was increased by resistin. The inhibitory effect of apelin on resistin-induced protein synthesis increase was blocked by HNE (Fig. 2).

Apelin decreases expression of BNP and β-MHC mRNA that is increased by resistin: To investigate the effect of apelin on expression of BNP and β-MHC mRNA induced by resistin which are cardiomyocyte hypertrophy markers, H9c2 cells were treated with resistin, apelin and HNE alone or combination. Resistin treatment increased expression of BNP and β-MHC in mRNA levels. Apelin treatment suppressed resistin-induced

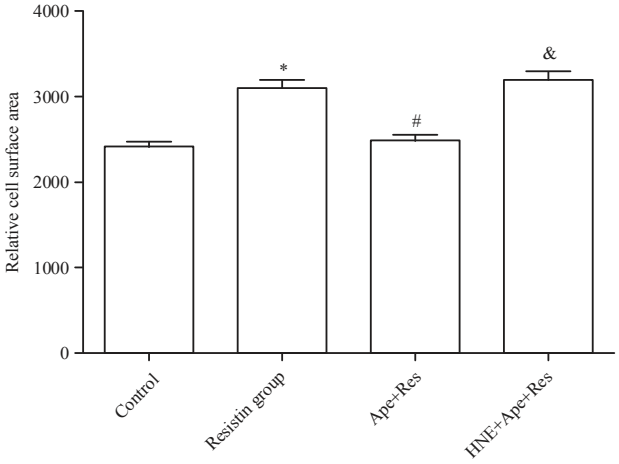

Fig. 1: Effect of apelin on cell surface area increased by resistin. Cell surface area was measured using NIH ImageJ software. Data represent Mean±SD. *p<0.01 vs. the control group, #p<0.01 vs. the resistin group, &p<0.01 vs. the apelin+resistin group
Res: Resistin group, Ape+Res: Apelin+resistin group, HNE+Ape+Res: HNE+apelin+resistin group

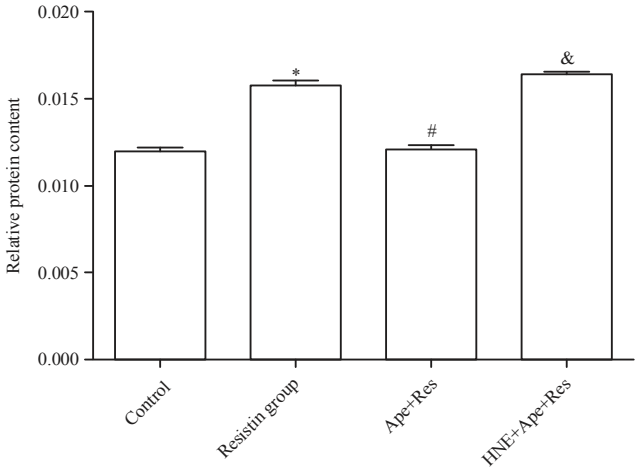

Fig. 2: Effect of apelin on protein synthesis increased by resistin. Cell protein contents were measured and normalized to the cell number. Data represent Mean±SD. *p<0.01 vs. the control group, #p<0.01 vs. the resistin group, &p<0.01 vs. the apelin+resistin group

increase of BNP and β-MHC mRNA expression. The HNE treatment reversed the effect of apelin that inhibited BNP and β-MHC mRNA expression induced by resistin (Fig. 3).

Apelin increases phosphorylation of LKB1 and AMPK that is decreased by resistin: To understand the underlying

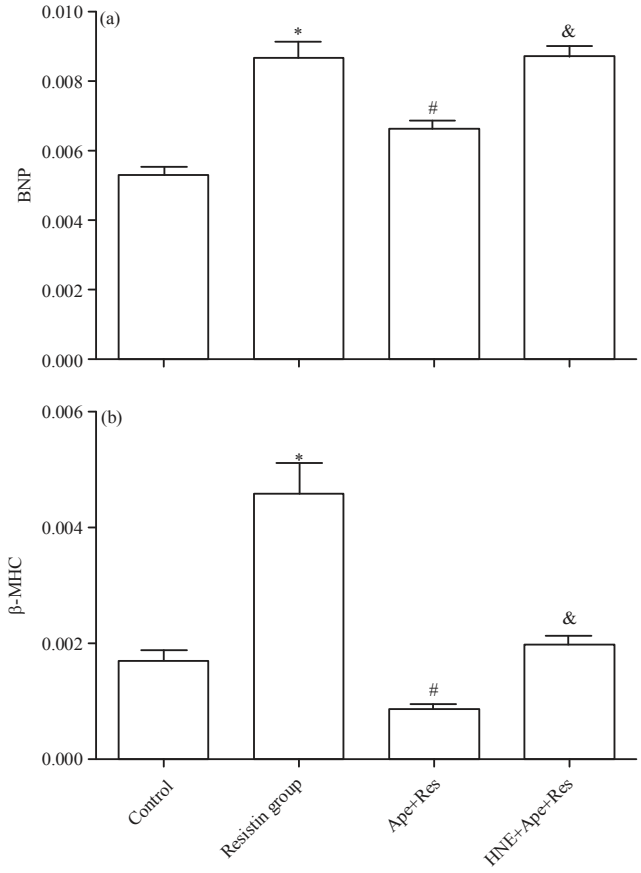

Fig. 3(a-b): Effect of apelin on BNP and β-MHC mRNA expression induced by resistin, (a) BNP mRNA and (b) β-MHC mRNA levels were examined by RT-qPCR. Data represent Mean±SD. *p<0.01 vs. the control group, #p<0.01 vs. the resistin group, &p<0.01 vs. the apelin+resistin group

molecular mechanism by which apelin inhibits cardiomyocyte hypertrophy induced by resistin, western blot was used to detect phosphorylation of LKB1 and AMPK. Treatment of resistin decreased phosphorylation of LKB1 and AMPK, whereas total LKB1 and AMPK protein expression was not in changed. Pre-treatment of apelin increased expression of phosphorylated LKB1 and AMPK that was decreased by resistin. In addition, resistin-induced phosphorylation of LKB1 and AMPK decrease was increased by apelin treatment and this could be reversed by HNE treatment (Fig. 4a-c).

DISCUSSION

Recent experiments displayed that treatment of resveratrol inhibits cardiac hypertrophy through decreased HNE-LKB1 adduct formation and activated LKB1/AMPK

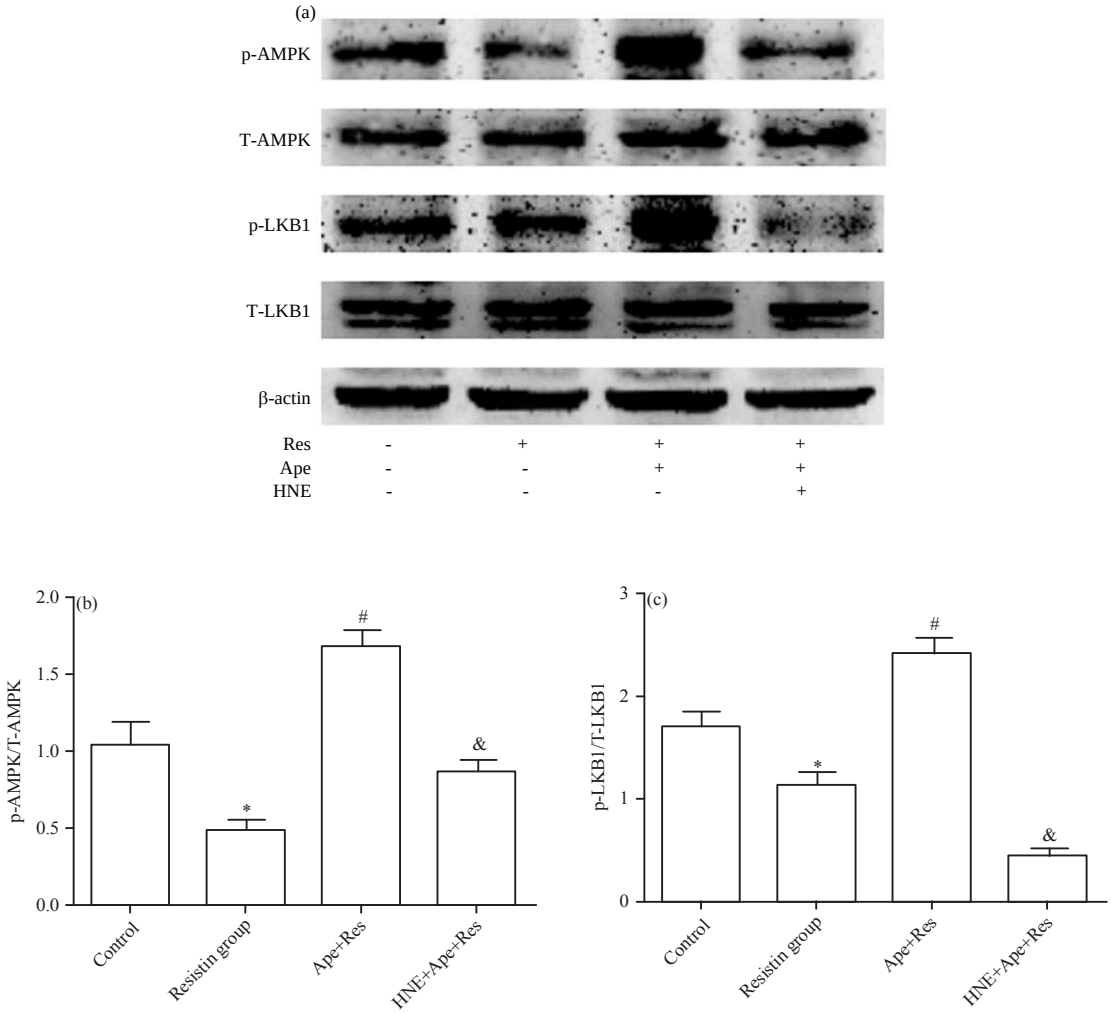

Fig. 4(a-c): Effect of apelin on phosphorylated LKB1 and AMPK expression decreased by resistin, (a) Western blot, (b) Expression of p-AMPK was normalized with AMPK and (c) Expression of p-LKB1 was normalized with LKB1. Data represent Mean±SD. *p<0.01 vs. the control group, #p<0.01 vs. the resistin group, &p<0.01 vs. the apelin+resistin group

signaling pathway[22]. The HNE is the major cytotoxic aldehyde generated during lipid peroxidation[21]. The HNE can covalently modify the LKB1 protein to form HNE-LKB1 adduct and inhibit the LKB1 activity and subsequent the AMKP activity. Treatment of cardiomyocytes with HNE results in significantly reduced levels of P-LKB1 and P-AMPK and increased cardiomyocyte hypertrophy[23]. In the experiments, resistin increased cell surface area, protein synthesis and hypertrophic marker BNP and β-MHC mRNA expression, whereas apelin suppressed these effects of resistin, which was blocked by HNE, suggesting that resistin-induced cardiomyocyte hypertrophy can be inhibited by apelin.

Resistin is an adipokine that potentially links obesity to diabetes. Treatment of normal mice with recombinant resistin impairs glucose tolerance and insulin action[3]. Many factors can regulate the secretion of resistin, such as endothelin-1[5], metformin[24] and glucose[8]. Overexpression of resistin *in vivo* using adeno-associated virus serotype 9 significantly decreases left ventricular contractility and induces oxidative stress, inflammation, fibrosis, apoptosis and myocardial remodeling in normal rats[4]. Resistin levels are associated with cardiac hypertrophy in the study of patients with hypertrophic cardiomyopathy[25]. Overexpression of resistin induces cardiac hypertrophy in neonatal rat cardiomyocytes through activation of the oxidative stress, IRS1/MAPK[26], AMPK/mTOR/p70S6K and ASK1/JNK/IRS1 signaling pathways[27]. In this study, H9c2 cells were used as a model, treatment with resistin induced cardiomyocyte hypertrophy. Treatment with resistin also decreased LKB1 and AMPK phosphorylation, suggesting that resistin induces cardiomyocyte hypertrophy through the inactivation of LKB1/AMPK signaling pathway.

Apelin is an adipokine which is endogenous ligand for the APJ receptor. Apelin has extensive role in cardiovascular system. Apelin increases cardiac contractility via protein kinase Cε and extracellular signal-regulated kinase-dependent mechanisms and protects heart against ischemia-reperfusion injury through inhibition of RISK-GSK-3β-mPTP pathway[28]. Apelin behaves as a catalase activator and prevents cardiac ROS-dependent hypertrophy[29]. Apelin decreases left ventricular preload and after load and increases contractile reserve[30].

Exogenous apelin-13 attenuates cardiac dysfunction and remodeling and restores apelin/APJ expression in Dahl salt-sensitive hypertensive (DS) rats with end-stage heart failure (HF)[31]. In the study, resistin induced cardiomyocyte hypertrophy and decreased phosphorylation of LKB1 and AMPK. However, apelin inhibited cardiomyocyte hypertrophy induced by resistin. Meanwhile, apelin increased phosphorylation of LKB1 and AMPK that are decreased by resistin. The inhibitory effect of apelin on resistin was blocked by HNE. It turned out that apelin suppresses resistin-induced myocyte hypertrophy through the activation of LKB1/AMPK signaling pathway.

CONCLUSION

Resistin induced increased BNP and β-MHC mRNA expression, cell surface area and protein synthesis as well as decreased LKB1 and AMPK phosphorylation, while apelin inhibited these effects of resistin, which was further reversed by HNE, so this study suggested that apelin can inhibit resistin-induced cardiomyocyte hypertrophy through the activation of LKB1/AMPK signaling pathway. Current results give a new insight that apelin may be useful in the treatment of cardiac hypertrophy.

SIGNIFICANCE STATEMENT

This study discovered that apelin inhibits resistin-induced cardiomyocyte hypertrophy via the activation of LKB1/AMPK signaling pathway that gives a new insight that apelin may be useful in the treatment of cardiac hypertrophy. This study will help the researchers to explore the roles of more adipokines on cardiac hypertrophy.

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