LKB1/AMPK pathway mediates resistin-induced cardiomyocyte hypertrophy in H9c2 embryonic rat cardiomyocytes

PENG LIU[1], GUAN-CHANG CHENG[2], QUN-HUI YE[2], YONG-ZHI DENG[1] and LIN WU[2]

[1]Department of Cardiovascular Surgery, The Affiliated Cardiovascular Hospital of Shanxi Medical University, and Shanxi Cardiovascular Hospital (Institute), Taiyuan, Shanxi 030024; [2]Department of Cardiology, Huaihe Hospital of Henan University, Kaifeng, Henan 475000, P.R. China

Received September 24, 2015; Accepted January 27, 2016

DOI: 10.3892/br.2016.593

Abstract. Resistin has been previously demonstrated to induce cardiac hypertrophy, however, the underlying molecular mechanisms of resistin-induced cardiac hypertrophy remain unclear. Using H9c2 cells, the present study investigated the liver kinase B1 (LKB1)/adenosine monophosphate-activated protein kinase (AMPK) signaling pathway for a potential role in mediating resistin-induced cardiomyocyte hypertrophy. Treatment of H9c2 cells with resistin increased cell surface area, protein synthesis, and expression of hypertrophic marker brain natriuretic peptide and β-myosin heavy chain. Treatment with metformine attenuated these effects of resistin. Furthermore, treatment with resistin decreased phosphorylation of LKB1 and AMPK, whereas pretreatment with metformin increased phosphorylation of LKB1 and AMPK that is reduced by resistin. These results suggest that resistin induces cardiac hypertrophy through the inactivation of the LKB1/AMPK cell signaling pathway.

Introduction

Cardiac hypertrophy is an adaptive response to stimulation to heart, eventually progressing to heart failure. Features of cardiac hypertrophy include increased cardiomyocyte size, protein synthesis, elevated fetal gene atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), β-myosin heavy chain (β-MHC) and fibronectin protein expression, and abnormal sarcomeric organization. Cell signaling pathways involved in cardiac hypertrophy include mitogen-activated protein kinase (MAPK), adenosine monophosphate-activated protein kinase (AMPK), transforming growth factor β (TGF-β)/smads, Ras/Rho, janus kinase (JAK)/signal transducers and activators of transcription (STAT), and calcinurin/nuclear factor of activated T-cells (NFAT) (1,2).

LKB1 is a signaling protein (3,4) that forms a complex with sterile-20-related adaptor (STRAD) and mouse protein-25 (MO25). STRAD and MO25 are required for the activity of LKB1 (5,6). AMPK is a substrate of LKB1, and is composed of AMPKα, AMPKβ and AMPKγ subunits. AMPKα is a catalytic subunit, composed of AMPKα1 and AMPKα2. AMPKβ and AMPKγ are regulatory subunits. AMPKα1 is widely expressed, whereas AMPKα2 is primarily expressed in liver cells and skeletal and cardiac muscle (7). The LKB1/AMPK pathway mediates various biological functions, including proliferation, apoptosis, autophagy and transcription (8,9).

Resistin is an adipocyte-secreted cytokine that is linked to obesity, diabetes, insulin resistance, and cardiac hypertrophy (10,11). Treatment of wild-type mice with resistin causes glucose intolerance (12), and immunoneutralization of resistin in obese mice decreases insulin sensitivity (10). Resistin can be regulated by a number of cytokines, including endothelin (ET), insulin, insulin-like growth factors (IGFs), and peroxisome proliferator-activated receptor (PPAR). Furthermore, resistin has been reported to induce cardiac hypertrophy through a number of signaling pathways, such as extracellular signal-regulated kinases (ERK), AMPK/mammalian target of rapamycin (mTOR), and the c-Jun NH (2)-terminal kinase (JNK)/insulin receptor substrate 1 (IRS1) pathway (11,13). The underlying molecular mechanisms by which resistin induces cardiac hypertrophy are still not completely understood.

The aim of the present study was to investigate the effects of resistin on LKB1/AMPK cell signaling and the induction of cardiac hypertrophy in the H9c2 rat myoblast cell line.

Materials and methods

Reagents. Recombinant human resistin was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). Metformin was ordered from Sigma-Aldrich (St. Louis, MO, USA). The H9c2 rat cardiomyoblast cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Fetal calf serum (FCS) was

Correspondence to: Professor Yong-Zhi Deng, Department of Cardiovascular Surgery, The Affiliated Cardiovascular Hospital of Shanxi Medical University, and Shanxi Cardiovascular Hospital (Institute), Taiyuan, Shanxi 030024, P.R. China
E-mail: 13834591065@163.com

Professor Lin Wu, Department of Cardiology, Huaihe Hospital of Henan University, Kaifeng, Henan 475000, P.R. China
E-mail: linwu71ch@yahoo.com

Key words: resistin, cardiac hypertrophy, liver kinase B1, adenosine monophosphate-activated protein kinase, cell signaling pathway

purchased from Zhejiang Tianhang Biological Technology (Zhejiang, China). Antibodies raised against phospho-LKB1, LKB, phospho-AMPK and AMPK were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The UNIQ-10 column TRIzol® 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% FCS, 1% penicillin and 1% streptomycin at a temperature of 37˚C with a 5% CO_2 atmosphere. Once cells had reached 70-80% confluence, they were passaged according to a 1:2 or 1:3 proportion. The medium was changed every 2 days. Cells were seeded at a density of $1x10^5$ into a 35 mm tissue culture dish. Cells were cultured in serum-free medium overnight and treated with resistin at a concentration of 50 ng/ml for the indicated time.

Determination of cell surface area. In total, $8x10^4$ cells were seeded in a 35-mm dish. Cells were cultured with serum-free DMEM overnight and treated with resistin for 48 h. The cell surface area was determined via quantification of the total surface (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 area (14).

Protein synthesis measurement. In total, $1x10^5$ cells were seeded in a 35-mm dish. Cells were cultured with serum-free DMEM overnight and treated with resistin for 48 h. Cells were digested with trypsin and counted under a microscope. The cells were then collected and lysed in 100 μl of RIPA buffer. Protein concentrations were measured using the Bradford protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell protein synthesis was determined by dividing the total amount of protein by the cell number (15). The trypan blue exclusion test of cell viability was used to determine the number of viable cells present in a cell suspension.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cells were collected and RNA was extracted using the UNIQ-10 column TRIzol kit (Shanghai Sangon Biotech) 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 conditions were: 95˚C for 30 sec, then 40 cycles of 95˚C for 5 sec followed by 60˚C for 31 sec. 18s was used as a reference gene. The ΔΔCq method was used for relative quantification. The BNP, β-MHC and 18s primers were designed and synthesized by Sangon Biotech Co., Ltd. The nucleotide sequences of the primers were as follows: BNP forward, 5'-GGAGCATTGAGTTGGCTCTC-3' and reverse, 5'-CCAGCTCTCCGAAGTGTTTC-3'; β-MHC forward, 5'-CACCCGCGAGTACAACCTTC-3' and reverse, 5'-CCCATACCCACCATCACACC-3'; 18s forward, 5'-CACCCGCGAGTACAACCTTC-3' and reverse, 5'-CCCATACCCACCATCACACC-3'.

Western blot analysis. Once the cells has reached 80-90% confluence, they were washed twice with phosphate-buffered saline, digested with 0.05% trypsin (Beyotime Biotechnology, Beijing, China) for 1 min and centrifuged at 1,000 x g for 5 min. Cells were mixed with 100 μl of lysis buffer and incubated on ice for 20 min. Lysates were centrifuged at 1,000 x g and protein concentration was measured using the bicinchoninic acid assay. 5X Laemmli's buffer was added to samples, which were then heated at 95˚C for 5 min and then separated by 10% 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. Following primary antibody 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 an enhanced chemiluminescence kit (ComWin Biotech, Beijing, China) and FluorChem™Q Quantitative Western Blot Imaging System (Bio-Techne, Minneapolis, MN, USA). The band intensities were measured with ImageJ software, and the ratio of phosphorylated protein antibodies over corresponding total protein antibodies was calculated.

Statistical analysis. All experiments data were expressed as means ± SD and performed at least three times. The Student's t-test was used for statistical analysis and the differences were considered statistically significant if $P<0.05$.

Results

Resistin treatment increases H9c2 cell size, which is reversed by metformin. Resistin treatment was used to induce cardiomyocyte hypertrophy. H9c2 cells were treated with resistin at 50 mg/ml for 48 h. Resistin increased cell surface area significantly compared to the control group ($P<0.01$; Fig. 1). Pre-treatment of cardiomyocytes with metformin led to a significantly decreased resistin-induced cell surface area ($P<0.01$, Fig. 1).

Resistin increases the synthesis cardiomyocyte protein synthesis and opposes the effects of metformin treatment. To investigate whether resistin treatment increases protein synthesis in H9c2 cells and that this increase is inhibited by

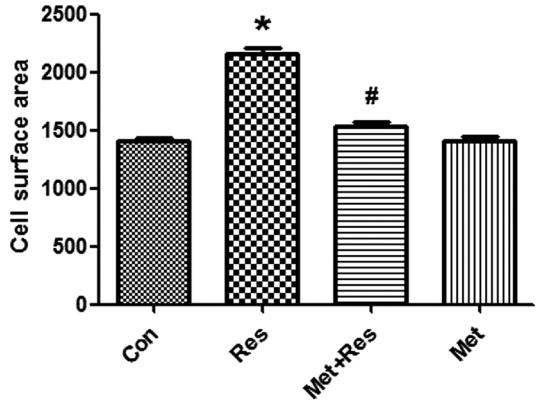

Figure 1. Resistin increases cell surface areas. Cell surface areas were measured using ImageJ software. Data represent the mean ± SD. *P<0.01 vs. the control group, #P<0.01 vs. the resistin group. Con, control; Res, resistin; Met+Res, metformin+resistin; Met, metformin.

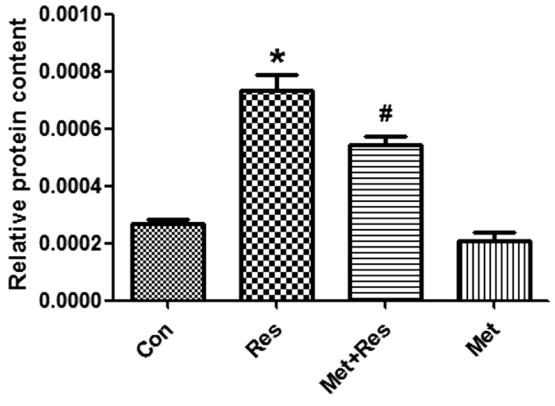

Figure 2. Resistin increases protein synthesis. Cell protein contents were measured and normalized to the cell number. Data represent the mean ± SD. *P<0.01 vs. the control group, #P<0.05 vs. the resistin group. Con, control; Res, resistin; Met+Res, metformin+resistin; Met, metformin.

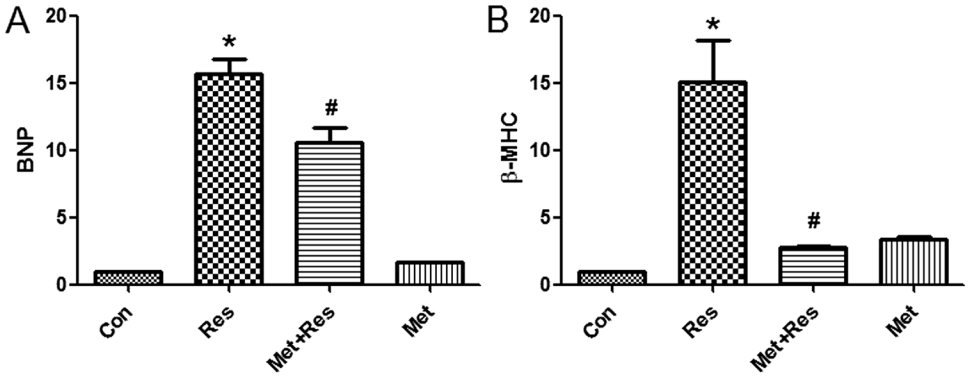

Figure 3. Resistin increases BNP and β-MHC mRNA expression that are reduced by metformin. (A) BNP mRNA and (B) β-MHC mRNA levels were examined by reverse transcription quantitative PCR. Data represent the mean ± SD. *P<0.01 vs. the control group, #P<0.05 vs. the resistin group. Con, control; Res, resistin; Met+Res, metformin+resistin; Met, metformin; BNP, brain natriuretic peptide; β-MHC, β-myosin heavy chain.

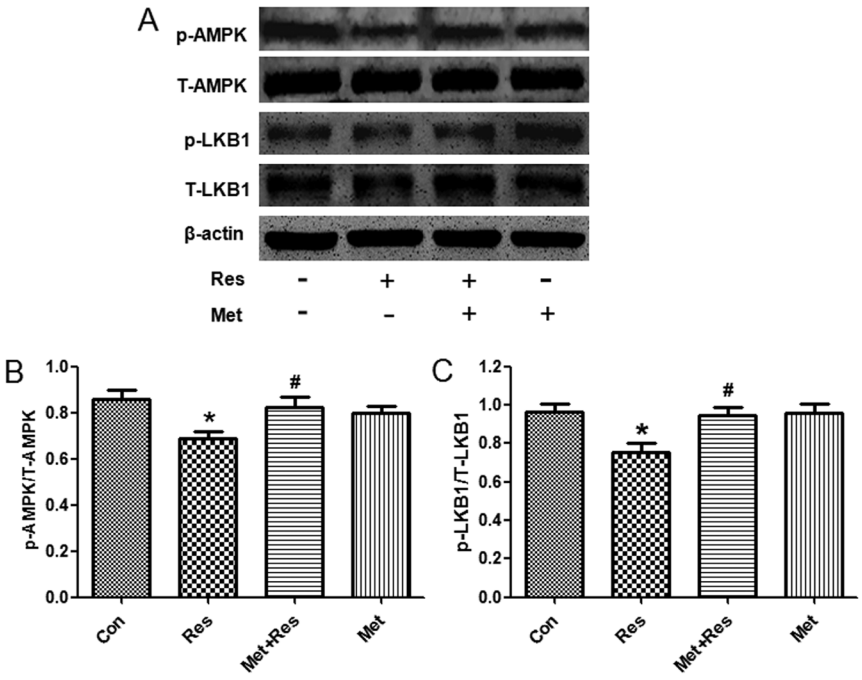

Figure 4. Resistin decreases phosphorylated liver kinase B1 (LKB1) and AMP-activated protein kinase (AMPK) expression. (A) Representive western blot. (B) The expression of p-AMPK normalized with AMPK. (C) The expression of p-LKB1 normalized with LKB1. Data represent the mean ± SD. *P<0.05 vs. the control group, #P<0.05 vs. the resistin group. Con, control; Res, resistin; Met+Res, metformin+resistin; Met, metformin.

metformin, cultured cardiomyocytes were exposed to resistin in the presence and/or absence of metformin for 48 h. The results demonstrate that resistin significantly increased protein synthesis in cardiomyocytes ($P<0.05$; Fig. 2). Furthermore, metformin treatment significantly decreased the synthesis of proteins that were increased by resistin ($P<0.05$; Fig. 2).

Resistin treatment elevates the mRNA expression levels of BNP and β-MHC. As BNP and β-MHC are markers of cardiomyocyte hypertrophy, the effects of resistin treatment on the expression of BNP and β-MHC mRNA was investigated in H9c2 cells. The results demonstrate that resistin treatment increased the expression of BNP and β-MHC mRNA. Additionally, the results identified that metformin treatment suppressed resistin-induced increase of BNP and β-MHC mRNA expression (Fig. 3).

Resistin treatment reduces the phosphorylation of LKB1 and AMPK. To further investigate the underlying molecular mechanism by which resistin induces cardiac hypertrophy, we used western blot analysis to evaluate the phosphorylation status of LKB1 and AMPK following resistin treatment. Treatment of resistin decreased phosphorylation of LKB1 and AMPK, whereas total LKB1 and AMPK protein expression was unchanged. Since metformin is an activator of LKB1 and AMPK (16,17), treatment of metformin increased expression of phosphorylated LKB1 and AMPK that was decreased by resistin (Fig. 4).

Discussion

Increasing evidence indicates that resistin induces cardiac hypertrophy. However, the underlying mechanisms by which resistin induces cardiac hypertrophy remain largely unknown. In the present study, resistin treatment increased cell size, protein synthesis and hypertrophic marker BNP and β-MHC mRNA expression, suggesting that resistin can induce cardiomyocyte hypertrophy. Resistin-induced cardiac hypertrophy may be mediated by LKB1/AMPK pathway.

Resistin is an adipocyte-secreted cytokine, released from fat cells in rodents (10). However, in humans, resistin is secreted from monocytes and macrophages (18-21). The function of resistin is related to obesity, diabetes and insulin resistance (10). Furthermore, treatment of resistin impairs glucose tolerance and insulin action, whereas loss of resistin function improves insulin resistance (22-24). Overexpression of resistin induces cardiac hypertrophy in neonatal rat cardiomyocytes through the activation of the oxidative stress (25), IRS1/MAPK (11), AMPK/mTOR/p70S6K and Apoptosis signal-regulating kinase 1 (ASK1)/JNK/IRS1 signaling pathways (13).

Liver kinase 1 is a tumor suppressor gene widely expressed in all tissues, and is involved in cell polarity, cell motility, protein translation, energy metabolism and various signal transduction pathways (26-29). LKB1 can phosphorylate downstream AMPK at threonine 172 (30-32). Metformin, phenformin and AICAR are activators of AMPK (4,33-35). The activation of AMPK has antiproliferative activity. The mTOR pathway is one of the downstream targets of AMPK, which is negatively regulated by LKB1/AMPK signaling. Inhibition of mTOR activity leads to inhibition of protein synthesis and proliferation (36). Furthermore, the LKB1-AMPK pathway plays an important role in cardiac hypertrophy development. It has been shown previously that 4-hydroxy-trans-2-nonenal (HNE) and miR-451 promote cardiac hypertrophy through suppression of the LKB1/AMPK pathway (37,38). By contrast, resveratrol or NAD prevents cardiac hypertrophy through enhancing the LKB1/AMPK signal transduction pathway (31,39,40). The current study demonstrates that resistin decreases phosphorylation of LKB1, and subsequently decreases phosphorylation of AMPK. Whereas metformin, an activator of LKB 1 and AMPK, increased phosphorylation of LKB1 that is decreased by resistin. Similarly, metformin increased expression of phosphorylated AMPK, is suppressed by resistin. These results suggest that resistin promotes cardiomyocyte hypertrophy via the LKB1/AMPK pathway.

In conclusion, resistin treatment elevates BNP and β-MHC mRNA expression, cell surface area and protein synthesis, and decreases the levels of LKB1 and AMPK phosphorylation, suggesting that resistin induces cardiac hypertrophy through inactivation of LKB1/AMPK signaling pathway. These results suggest that prevention of resistin may be useful in the treatment of cardiac hypertrophy.

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