Abstract. The recently identified adipocytokine omentin was previously found to be expressed mainly in human omental and visceral adipose tissues. As such, reduced plasma concentrations of omentin were revealed to be associated with increased risks of cardiovascular diseases. Omentin has also been previously demonstrated to exert anti-inflammatory effects. By contrast, resistin is a protein that has been associated with obesity and type-2 diabetes mellitus, and the serum concentration of resistin is increased significantly in these populations. Resistin is involved in mediating inflammation development, where they can promote cardiac hypertrophy in humans through toll-like receptor 4 (TLR4)-related signaling. In the present study, the potential effects of omentin on resistin-induced hypertrophy in H9c2 cardiomyoblasts were investigated. In the absence/presence of omentin, H9c2 cardiomyoblasts were treated with resistin. Omentin was found to significantly inhibit resistin-induced increases in the surface area of H9c2 cardiomyoblasts as determined by immunofluorescence. In addition, omentin significantly inhibited resistin-induced increases in the mRNA expression of atrial natriuretic factor, brain natriuretic peptide, β-myosin heavy chain (which is a characteristic feature of cardiac hypertrophy) and TLR4, which was determined using reverse-transcription-quantitative PCR. According to western blotting results, omentin significantly inhibited resistin-induced ERK phosphorylation, which is an important mediator of cardiomyoblast hypertrophy. Furthermore, omentin significantly inhibited resistin-induced protein expression of TLR4, myeloid differentiation factor 88 (MyD88) and NF-κB phosphorylation, both of which are important members of inflammatory signaling. To conclude, data from the present study suggest that omentin can inhibit resistin-induced H9c2 cardiomyoblast hypertrophy through inhibition of the TLR4/MyD88/NF-κB signaling pathway. Therefore, omentin serve as an attractive therapeutic target against resistin-induced cardiac hypertrophy.

Introduction

It has been previously reported that ~66% of the mortality cases associated with diabetes mellitus (DM) can be attributed to cardiovascular diseases (1). In the absence of changes in blood pressure and coronary artery disease, DM can alter the structure and function of the heart by causing a condition known as ‘diabetic cardiomyopathy’ (2). Initially, diabetic cardiomyopathy is characterized by cardiac muscle hypertrophy and associated diastolic dysfunction, which is typically followed by systolic dysfunction and ultimately heart failure (3). A maladaptive inflammatory response has been implicated in the occurrence of cardiac hypertrophy during diabetic cardiomyopathy (4). Previous studies have revealed that alleviating the inflammatory response during cardiac hypertrophy is beneficial to the survival of diabetic cardiomyopathy (5,6).

The toll-like receptor 4 (TLR4)/NF-κB signaling pathway serves a key role in cardiac hypertrophy (7). Activity of this pathway was previously found to be significantly promoted in the hypertrophic myocardium (8). Gao et al (9) previously reported that inhibition or knockdown of TLR4/myeloid differentiation primary response 88 (MyD88) signaling pathway attenuated inflammatory and hypertrophic responses in transverse aortic constriction or angiotensin-II infusion of mice and cardiomyocytes isolated from mouse neonatal ventricles.

Resistin is a cysteine-rich polypeptide that is mainly secreted by macrophages in humans and by adipose tissues in rodents and humans (10). It has been reported to be positively
associated with obesity and the development of type-2 diabetes mellitus (T2DM) (10). In addition, serum levels of resistin were significantly higher in obese and T2DM patients compared with those in healthy subjects (11,12). Resistin has been previously found to be involved in mediating inflammation, insulin resistance, cardiac hypertrophy, hypertension, atherosclerosis, coronary artery disease and rheumatic diseases (13-17). Kim et al (14) previously found that resistin overexpression could decrease myocardial contractility, in addition to endowing primary cardiomyoblasts with hypertrophic phenotypes to promote cardiac hypertrophy. Other studies have also found that resistin can induce inflammation, insulin resistance and hypertension through a TLR4-dependent signaling pathway (13,15).

Omentin, also known as intelectin-1, is a cytokine that is typically secreted by the adipose tissue (adipocytokine). It is mainly expressed in omental and visceral adipose tissues in humans (18). Physiologically, omentin has been found to exhibit various pharmacological effects in the cardiovascular system, with protective effects against vascular inflammation (19), atherogenesis (20) and myocardial ischemia (21) among those reported. In addition, reduced circulating levels of omentin have been associated with increased risk of obesity-related diseases, including metabolic syndrome and T2DM (22). Therefore, these previous findings of omentin aforementioned suggest that it may serve a protective role against cardiovascular disorders associated with metabolism. However, the mechanistic role of omentin in resistin-induced cardiac hypertrophy remains poorly understood.

In the present study, the potential effects of omentin on resistin-induced hypertrophy in H9c2 cardiomyoblasts were investigated. The present study will investigate the relationship of omentin, resistin and TLR4/MyD88/NF-κB pathway through H9c2 cardiomyoblasts related experiments.

Materials and methods

Materials. Recombinant human omentin protein (cat. no. RD172100025) was purchased from BioVendor. Recombinant human resistin protein (450-19-25) was obtained from PeproTech, Inc. Anti-TLR4 antibody (cat. no. ab95562) was purchased from Abcam. Antibodies against MyD88 (cat. no. 4283), NF-κB p65 (cat. no. 8242), phosphorylated (p)-NF-κB p65 (cat. no. 3033), β-tubulin (cat. no. 2146), p-ERK (Thr-202/Tyr-204; cat. no. 4370), and ERK (cat. no. 4695) were obtained from Cell Signaling Technology, Inc. Antibody against β-actin (cat. no. SAB3500350) was from Sigma-Aldrich; Merck KGaA.

Culture of H9c2 cardiomyoblasts. H9c2 rat cardiomyoblasts were obtained from American Type Culture Collection. H9c2 cells were cultured in DMEM (Wako Pure Chemical Industries, Ltd.) containing 10% FBS (Zhejiang Tianhang Biotechnology, Co., Ltd.) and 1% penicillin-streptomycin in an atmosphere of 5% CO2 at 37°C, consistent with protocols described in a previous study (25). After the H9c2 cardiomyoblasts reached 90% confluence, they were growth-arrested in FBS-free medium for 24 h before stimulation with omentin or resistin at 37°C.

Immunofluorescence to ascertain the surface area of H9c2 cardiomyoblasts. After the H9c2 cardiomyoblasts were cultured in serum-free medium for 24 h, they were treated with resistin (100 ng/ml) for 48 h with or without omentin (3, 30 and 300 ng/ml; 1 h) pre-treatment (26-28) at 37°C. To determine the extent of α-actin organization within sarcomeres, cultured H9c2 cardiomyoblasts were fixed in 100% methanol for 10 min at 4°C, washed with PBS three times and blocked with 10% normal goat serum (Shanghai Yisheng Biotechnology, Co., Ltd.) for 30 min at room temperature. The cardiomyoblasts were then incubated with the mouse anti-α-sarcomeric actin primary monoclonal antibody (1:500; cat. no. 113200; Sigma-Aldrich; Merck KGaA) for 1 h at room temperature, followed by incubation with the FITC-conjugated secondary antibody (1:1,000; cat. no. sc-2359; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature (29). The nuclei of the H9c2 cardiomyoblasts were stained using Hoechst 33258 (1 μg/ml; Sigma-Aldrich; Merck KGaA) for visualization for 1 h at 37°C. A fluorescence microscope (Carl Zeiss, AG) was used to image the samples. The surface area of H9c2 cardiomyoblasts was measured using ImageJ 1.49 (National Institutes of Health) from two-dimensional images of 50 cells selected at random in 20 fields at x400 magnification.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the H9c2 cardiomyoblasts using TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer protocols. The RNA concentration was measured using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Inc.). Reverse transcription was performed using a complementary DNA (cDNA) reverse transcription kit PrimeScript RT Master Mix (cat. no. RR036Q; Takara Bio, Inc.). The conditions of reaction were: 37°C for 15, 85°C for 5 sec. The obtained cDNA was then subjected to qPCR for measurement of mRNA expression of atrial natriuretic peptide (ANF), B-type natriuretic peptide (BNP), β-myosin heavy chain (β-MHC) and TLR4 using a TB Green Premix Ex Taq II (cat. no. RR820Q; Takara Bio, Inc.).

YAN et al: OMENTIN INHIBITS H9C2 CARDIOMYOBLAST HYPERTROPHY
Western blotting. After the H9c2 cardiomyoblasts were treated with resistin (100 ng/ml) for 1 or 12 h with or without pretreatment with omentin (300 ng/ml; 1 h) at 37˚C, samples of total protein were obtained by homogenizing H9c2 cardiomyoblasts with RIPA lysis buffer (CoWin Biosciences). Protein concentration was determined using the bicinchoninic acid assay before 5X Laemmli buffer was added. An equal amount of protein (25-30 µg) was separated by 10% SDS-PAGE, before the proteins were transferred onto PVDF membranes (MilliporeSigma). After blockade with 0.5% non-fat milk or 5% bovine serum albumin (Boster Biological Technology) for 2 h at room temperature, the PVDF membranes were incubated with primary antibodies [TLR4, MyD88, phospho-(p)-NF-κB p65, NF-κB p65, p-ERK and ERK antibodies at 1:1,000 dilution; β-tubulin and β-actin at 1:2,000 dilution] overnight at 4˚C. After washing three times with TBS containing 0.1% Tween 20, the PVDF membranes were incubated with HRP-conjugated secondary antibodies (1:2,000 dilution; cat. no. 70745; Cell Signaling Technology, Inc.) for 1 h at room temperature. Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Bio-Techne) and the densitometry was quantified by ImageJ 1.49 (National Institutes of Health).

Statistical analysis. Data are presented as the mean ± SEM at least 4 independent experiments. Statistical comparisons were performed using one-way ANOVA followed by Bonferroni’s test. P<0.05 was considered to indicate a statistically significant difference. Statistical analyses were conducted using Graphpad Prism 5 (GraphPad Software, Inc.).

Results

Effects of omentin on the surface area of resistin-induced H9c2 cardiomyoblasts. Resistin has been previously shown to induce cardiomyoblast hypertrophy (14). In the present study, it was observed that resistin (100 ng/ml) significantly increased the surface area of the H9c2 cardiomyoblasts (1.69±0.12-fold relative to control; P<0.01; Fig. 1). Therefore, the potential effects of omentin (3, 30 and 300 ng/ml, 1 h) pre-treatment on this increase in the surface area of resistin-induced H9c2 cardiomyoblasts were examined. Omentin (300 ng/ml, 1 h) was found to significantly reverse the resistin (100 ng/ml, 48 h)-induced increases in the surface area of H9c2 cardiomyoblasts (300 ng/ml omentin + resistin, 1.16±0.14-fold relative to control; P<0.05; Fig. 1).

Effects of omentin on the mRNA expression of ANF, BNP, β-MHC and TLR4 in resistin-induced H9c2 cardiomyoblasts. Re-activation of fetal genes ANF, BNP and β-MHC is a characteristic feature of cardiac hypertrophy (31). Therefore, the present study next assessed the potential effects of omentin (300 ng/ml, 1 h) on the mRNA expression of ANF, BNP and β-MHC in resistin-induced H9c2 cardiomyoblasts. In addition, the effect of omentin (300 ng/ml; 1 h) on resistin-induced mRNA expression of TLR4 was also assessed in H9c2 cardiomyoblasts. Omentin...
YAN et al.: OMENTIN INHIBITS H9c2 CARDIOMYOBLAST HYPERTROPHY

significantly reversed the resistin-induced (100 ng/ml, 24 h) increase in expression of ANF mRNA (resistin, 1.57±0.07-fold relative to control; omentin + resistin, 1.21±0.06-fold relative to control; P<0.05; Fig. 2A). Omentin also significantly reversed the resistin-induced (100 ng/ml, 24 h) increase in expression of BNP mRNA (resistin, 1.79±0.09-fold relative to control; omentin + resistin, 1.25±0.10-fold relative to control; P<0.01; Fig. 2B). In addition, omentin significantly reversed the resistin-induced (100 ng/ml, 24 h) increase in expression of β-MHC mRNA (resistin, 1.95±0.18-fold relative to control; omentin + resistin, 1.35±0.11-fold relative to control; P<0.05; Fig. 2C). Omentin significantly reversed the resistin-induced (100 ng/ml, 24 h) increase in expression of TLR4 mRNA (resistin, 1.55±0.09-fold relative to control; omentin + resistin, 1.11±0.05-fold relative to control; P<0.05; Fig. 2D).

Effects of omentin on ERK phosphorylation in resistin-induced H9c2 cardiomyoblasts. An important process in cardiomyoblast hypertrophy is ERK activation (28,29). The present study next investigated the effects of omentin (300 ng/ml, 1 h) on resistin-induced ERK phosphorylation in H9c2 cardiomyoblasts by western blotting. Stimulation of H9c2 cardiomyoblasts with resistin (100 ng/ml, 24 h) had no effects on the protein expression of total ERK, but significantly increased phosphorylation levels of ERK at Thr-202/Tyr-204 and the p-ERK/ERK ratio (Fig. 3), suggesting an increase in ERK activity due to resistin. However, omentin pre-treatment significantly prevented the resistin-induced ERK phosphorylation (resistin, 2.48±0.19-fold relative to control; omentin + resistin, 1.64±0.20-fold relative to control; P<0.05; Fig. 3).

Figure 2. Effects of omentin on the mRNA expression of ANF, BNP, β-MHC and TLR4 in resistin-induced H9c2 cardiomyoblasts. After the H9c2 cardiomyoblasts were treated with resistin (100 ng/ml, 24 h) in the absence or presence of omentin (300 ng/ml, 1 h), total RNA was then extracted. (A) Expression of ANF, (B) BNP, (C) β-MHC and (D) TLR4 mRNA was measured by reverse transcription-quantitative PCR. n=4. **P<0.01 vs. control; *P<0.05 and ##P<0.01 vs. resistin-only. ANF, atrial natriuretic peptide; BNP, B-type natriuretic peptide; β-MHC, β-myosin heavy chain; TLR4, toll-like receptor 4.

Figure 3. Effects of omentin on ERK activation in resistin-induced H9c2 cardiomyoblasts. After the H9c2 cardiomyoblasts were treated with resistin (100 ng/ml, 1 h) in the absence or presence of omentin (300 ng/ml, 1 h), cardiomyoblast protein lysates were obtained. Phosphorylation of ERK was measured by western blotting. n=4. **P<0.01 vs. Control; *P<0.05 vs. Resistin-only. p-, phosphorylated.
Effect of omentin on the protein expression of TLR4, MyD88 and NF-κB p65 phosphorylation in resistin-induced H9c2 cardiomyoblasts. The present study next assessed the effects of omentin (300 ng/ml, 1 h) on the resistin-induced protein levels of TLR4, MyD88, p-NF-κB p65 and NF-κB p65 in H9c2 cardiomyoblasts by western blotting. Omentin significantly inhibited the resistin-induced (100 ng/ml, 12 h) protein expression of TLR4 (resistin, 1.70±0.07-fold relative to control; omentin + resistin, 1.28±0.09-fold relative to control, P<0.05; Fig. 4B) in H9c2 cardiomyoblasts. Omentin also significantly prevented the resistin-induced (100 ng/ml, 12 h) expression of the MyD88 protein (resistin, 2.03±0.09-fold relative to control; omentin + resistin, 1.44±0.17-fold relative to control, P<0.05; Fig. 4C) in H9c2 cardiomyoblasts. In addition, omentin significantly reversed the resistin-induced (100 ng/ml, 12 h) protein phosphorylation of NF-κB p65 (resistin, 1.86±0.17-fold relative to control; omentin + resistin, 1.21±0.14-fold relative to control, P<0.05; Fig. 4D) in H9c2 cardiomyoblasts. However, omentin had no effects on the protein expression of total NF-κB p65, suggesting that omentin inhibited the activity of resistin-induced TLR4/MyD88/NF-κB signaling in H9c2 cardiomyoblasts.

Discussion

In the present study, it was demonstrated that omentin inhibited the resistin-induced hypertrophy of H9c2 cardiomyoblasts. In addition, omentin inhibited resistin-induced expression of TLR4, MyD88 and NF-κB p65 phosphorylation, which are important molecular components of the TLR4/MyD88/NF-κB inflammatory pathway (32). Omentin also inhibited the resistin-induced re-activation of the expression of fetal genes ANF, BNP and β-MHC, which is the characteristic feature of cardiac hypertrophy (31). Furthermore, it was demonstrated that omentin inhibited the resistin-induced activation of ERK, which is an important mediator of cardiomyoblast hypertrophy (33,34). Taken together, these observations suggest that omentin can inhibit the hypertrophy of resistin-induced H9c2 cardiomyoblasts by blunting the activity of TLR4/MyD88/NF-κB signaling.

TLRs are pathogen pattern-recognition receptors and serve as important components of the innate immune system (35). The first member of the TLR family to be identified was TLR4 (32), which can mediate myocardial inflammation (36). In addition, the TLR4-activated mediation of inflammatory signaling serves an important role in myocarditis, cardiac hypertrophy, ischemia-reperfusion injury and myocardial infarction (37-39). A number of studies have previously shown that production of proinflammatory factors is regulated by the TLR4/MyD88/NF-κB signaling pathway, which in turn induces inflammation in the myocardial tissues and causing injury (40,41). In addition, it has been reported that resistin is involved in myocardial inflammation through...
a TLR4-associated pathway to cause myocardial tissue injury (13-15,42,43).

Cardiac hypertrophy is an adaptive change of the myocardium to increase the volume or pressure loads (44). However, this physiological adaptation is frequently accompanied with pathological changes (31). Activation of several intracellular signaling pathways is closely associated with the occurrence and development of cardiac hypertrophy. Han et al (45) found TLR4 activation could initiate myocardial remodeling. In another study, Ehrentraut et al (46) previously found that TLR4 antagonists could reduce cardiac hypertrophy in mice. Activation of the TLR4/MyD88/NF-κB signaling pathway has been demonstrated to increase the expression of a number of the proinflammatory cytokines, such as TNF-α and IL-6, which involved in the inflammatory response to cause myocardial injury (40,41). In addition, cardiac hypertrophy and myocardial inflammation were stifled effectively by inhibiting the activity of the TLR4 signaling pathway (46,47). In particular, inhibiting the expression of NF-κB p65 which is one of components in the NF-κB signaling pathway was able to reduce the myocardial inflammatory response, inhibit the development of cardiac hypertrophy and reduce the risk of heart failure in the transgenic mouse (48). The present study demonstrated that resistin could significantly activate the TLR4/MyD88/NF-κB signaling pathway in H9c2 cardiomyoblasts, to induce an increase in the surface area of H9c2 cardiomyoblasts.

Omentin is a newly identified adipocytokine that was shown to exert an anti-inflammatory effect (49,50). Previous studies have demonstrated that various cardiovascular diseases, including carotid atherosclerosis and coronary artery disease, manifest with reduced plasma concentrations of omentin (23,24,51). Genre et al (52) found that low serum omentin levels were associated with cardiovascular risk factors, including obesity and high atherosclerosis indices, in patients with axial spondylarthritis. In addition, a number of previous studies have demonstrated high plasma concentrations of omentin to be associated with superior outcomes in patients with acute heart failure or coronary heart disease (49,53). Ma et al (8) found that inhibition of TLR4 pathway can inhibit myocardial hypertrophy in mice. In the present study, H9c2 cardiomyoblasts were first pre-treated with omentin (3, 30 and 300 ng/ml) and then found that 300 ng/ml omentin prevented the resistin-induced hypertrophy of H9c2 cardiomyoblasts. However, omentin at 3 and 30 ng/ml could not, which are consistent with the cardiovascular benefits of increasing the human serum omentin concentration (53). In addition, it was found that omentin inhibited the mRNA and protein expression of TLR4 induced by resistin in H9c2 cardiomyoblasts. Omentin also inhibited the protein expression of MyD88 and phosphorylation of NF-κB p65 after resistin stimulation in H9c2 cardiomyoblasts. Therefore, the ability of omentin to attenuate cardiomyoblast hypertrophy is likely due to its ability to inhibit the expression of components in the TLR4/MyD88/NF-κB signaling pathway in H9c2 cardiomyoblasts. Collectively, these data suggest that omentin-mediated inhibition of the TLR4/MyD88/NF-κB signaling pathway may represent a common pathway that leads to the beneficial actions of omentin in the cardiovascular system.

ERK activation is an important process in cardiomyoblast hypertrophy (33,34). Inhibition of TLR4 was shown to reduce ERK activity in various cell types, including cardiomyoblasts (44,45). This suggests that inhibition of TLR4 may reduce cardiomyoblasts hypertrophy. The present study demonstrated that stimulation of H9c2 cardiomyoblasts with resistin led to increased ERK activity. However, pre-treatment of the H9c2 cardiomyoblasts with omentin inhibited ERK activation in response to resistin treatment.

Reactivation of fetal myocardial genes, including ANF, BNP and β-MHC, is a characteristic feature of cardiac hypertrophy (31). The present study found that resistin could enhance the expression of these fetal myocardial genes in H9c2 cardiomyoblasts, whilst omentin could inhibit this resistin-induced expression of myocardial fetal genes in H9c2 cardiomyoblasts. Therefore, inhibition of resistin-induced cardiomyoblast hypertrophy by omentin may be associated with the inhibition of the expression of myocardial fetal genes.

A number of limitations are associated with the present study. The findings from the present study would require verification in vivo. In addition, agonists of the TLR4/MyD88/NF-κB signaling pathway or TLR4 knock-down would need to be used to clarify if the inhibition of omentin on resistin-induced hypertrophy of cardiomyoblasts can be reversed. The extent of p65 activation in the nucleus would also require further confirmation in subsequent studies. Furthermore, measurements of the expression of inflammatory factors following omentin treatment will need to be performed. It would also be of interest to investigate the potential effects of omentin on resistin-induced oxidative stress (27).

To conclude, the present study showed that omentin can inhibit resistin-induced hypertrophy of H9c2 cardiomyoblasts through inhibition of the TLR4/MyD88/NF-κB signaling pathway. These results suggest that omentin may be an attractive therapeutic target against resistin-induced cardiac hypertrophy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
Authors’ contributions

YD designed the current study. YD and XY wrote the manuscript. XY established the hypertrophic model of H9c2 cardiomyoblasts. XY and MG performed H9c2 cardiomyoblasts immunofluorescence staining. XY and JY performed RT-qPCR. LW and PY performed western blotting. XY performed statistical analysis. YD and XY confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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