Biochemical and Biophysical Research Communications 425 (2012) 401–406

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Omentin inhibits TNF-α-induced expression of adhesion molecules in endothelial cells via ERK/NF-κB pathway

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ARTICLE INFO

Article history:
Received 18 July 2012
Available online 27 July 2012

Keywords:
Omentin
ERK
NF-κB
ICAM-1
VCAM-1

ABSTRACT

In the present study, we investigated whether omentin affected the expression of intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in tumor necrosis factor-α (TNF-α) induced human umbilical vein endothelial cells (HUVECs). Our data showed that omentin decreased TNF-α-induced expression of ICAM-1 and VCAM-1 in HUVECs. In addition, omentin inhibited TNF-α-induced adhesion of THP-1 cells to HUVECs. Further, we found that omentin inhibited TNF-α-activated signal pathway of nuclear factor-κB (NF-κB) by preventing NF-κB inhibitory protein (IκBα) degradation and NF-κB/DNA binding activity. Omentin pretreatment significantly inhibited TNF-α-induced ERK activity and ERK phosphorylation in HUVECs. Pretreatment with PD98059 suppressed TNF-α-induced NF-κB activity. Omentin, NF-kB inhibitor (BAY11-7082) and ERK inhibitor (PD98059) reduced the up-regulation of ICAM-1 and VCAM-1 induced by TNF-α. These results suggest that omentin may inhibit TNF-α-induced expression of adhesion molecules in endothelial cells via blocking ERK/NF-κB pathway.

1. Introduction

Adipokines can influence carbohydrate and lipid metabolism, insulin resistance, atherosclerosis, vascular endothelial dysfunction and inflammation [1–5]. Omentin, a novel adipose tissue-derived adipokine, is negatively correlated with insulin resistance and obesity [6,7]. And omentin may also be a proinflammatory adipokine for having been implicated in a variety of chronic inflammatory diseases [8,9]. Our data [10] have shown that serum omentin-1 levels were lower in patients with coronary artery disease (CAD) compared with control participants. The patients with acute coronary syndrome (ACS) also had lower serum concentrations of omentin-1 compared to patients with stable angina pectoris (SAP).

Nuclear factor-κB (NF-κB) is a potent proinflammatory nuclear transcription factor which is included in the initiation and amplification of inflammatory responses [11]. NF-κB activation and DNA binding is dependent on degradation of its inhibitory protein (IκBα) and thus induces the transcription of target genes [12]. Numerous recent studies have demonstrated that NF-κB was involved in the development of inflammation, and metabolic disease [13]. The expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) play important roles in inflammatory processes and immune responses. There have been several studies showing that the adipokines such as visfatin, tumor necrosis factor-α (TNF-α) and adiponectin induced or reduced expression of inflammatory mediators including monocyte chemotactic protein-1 (MCP-1), IL-6, IL-8, E-selectin, ICAM-1 and VCAM-1 in human endothelial cells through NF-κB pathway [14–17]. However, the relationship about omentin, NF-κB, ICAM-1 and VCAM-1 is unclear.

All these observations prompted us to explore whether omentin had influence on the expression of cell adhesion molecules in TNF-α induced endothelial cells by affecting the activity of NF-κB as a downstream target. We found that omentin downregulated TNF-α-induced expression of adhesion molecules in endothelial cells by means of ERK-dependent pathway that finally led to the inhibition of NF-κB.

2. Materials and methods

2.1. Cell culture and treatments

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord with collagenase and used at passages 4-6. This study has been approved by the ethical committee of Provincial Hospital Affiliated to Shandong University and written informed consent was obtained. HUVECs were grown in medium 199 (Invitrogen, Carlsbad, CA) containing 20% fetal calf serum. Before the experiments the cells were shifted to to the same medium containing only 0.2% fetal calf serum, and were incubated overnight. For some experiments, HUVECs were treated with

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http://dx.doi.org/10.1016/j.bbrc.2012.07.110

omentin (300 ng/ml), the ERK inhibitor PD98059 (50 μM) or the NF-kB inhibitor BAY11-7082 (50 mM) for 1 h followed by administration of TNF-α (10 ng/ml) for 6 h.

2.2. Measurement of ICAM-1 and VCAM-1 in HUVECs by ELISA

The concentration of ICAM-1 and VCAM-1 in conditioned media was measured using Human ELISA Kits (Cusabio Biotech Corporation, USA), according to the manufacturer's instructions. Each experimental condition was tested in three different wells and measured in duplicate.

2.3. Adhesion assay

THP-1 cells (pre-labeled with 2,7 bis-(2-carboxyethyl)-5-(and-6) carboxy-fluorescein, acetoxymethyl ester 4 mM for 30 min in RPMI) was added to each 6-well plate containing endothelial cells for 1 h incubation. Non-adherent cells were removed by washing with medium 199. The number of adherent cells was estimated by microscopic examination, and then lysed with 0.1 ml of 0.25% Triton X-100. The fluorescence intensity was measured at 485 nm excitation and 538 nm emission using a fluorescence microplate reader (Leica, $DMIRE_2$, Germany).

2.4. Real-time PCR

Total RNA was extracted from cells using a standard Trizol RNA isolation method. Reverse transcription of 1 μg RNA was carried out according to the instructions of a commercial Takara RT kit (Takara, Japan). Quality of the RNA and cDNA was checked using DU640 nucleic-acid analyzer (Beckman Coulter, Fullerton, CA, USA). β-Actin primers were used as an internal control. Following sense and antisense primers were used, respectively: for ICAM-1, 5′-CGATGACCATCTACAGCTTTCCGG-3′ and 5′-GCTGCTACCACAGTGATGATGACAA-3′; for VCAM-1, 5′-GATACAACCGTCTTGGTC AGCC C-3′ and 5′-CAGTTGAAGGATGCGGGAG TATATG-3′; and for β-actin, 5′-GACTAC CTCATGAAGATC-3′ and 5′-GATCCACATCTGCTG GAA-3′; Real-time PCR was performed with a kit (Quantitect SYBR Green; Qiagen Inc.) following the manufacturer's instructions. The total reaction volume was 25 μl, and 100 ng cDNA was used as the template. Fluorescence was detected using a detection system (Prism 7700; ABI, Foster city, CA, USA). The C_t value was defined as the number of PCR cycles where the fluorescence signal exceeded the detection threshold value. Ratios of the target gene to β-actin were calculated and compared.

2.5. Western-blotting

Harvested cells were lysed in RIPA buffer containing 1× BS, 1% NP-40, 0.1% SDS, 5 mM ethylenediaminetetraacetic acid, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride. Cells were then centrifuged at 12,000*g* for 10 min at 4 °C. The supernatants were collected and frozen at −80 °C or used immediately. Protein concentrations were determined with a protein assay (bicinchoninic acid, BCA; Pierce, Rockford, IL, USA). We heated 40 μg of each sample for 30 min at 60 °C, then analyzed them by performing 8% SDS-PAGE and by electroblotting them onto nitrocellulose membranes. Membranes were blocked in 5% non-fat milk for 1 h and then incubated with primary antibodies overnight at 4 °C. Afterward, they were washed and incubated with appropriate horseradish peroxidase-conjugated secondary antibody. Immune complexes were detected using the

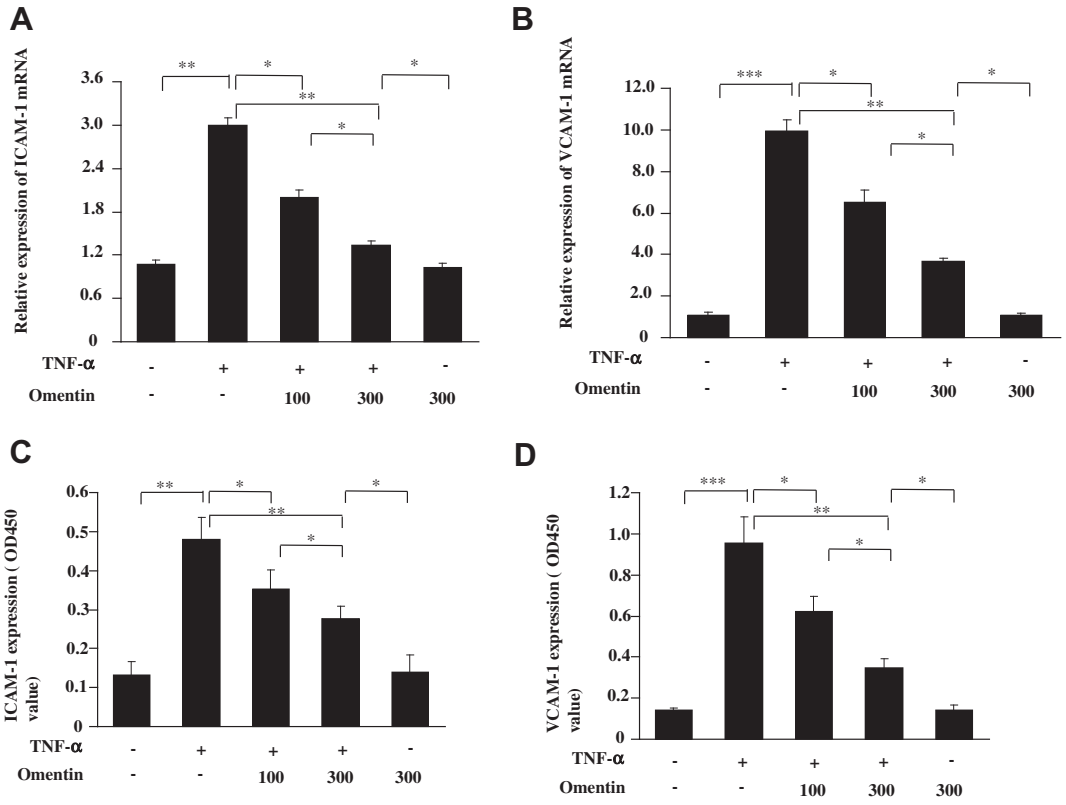

Fig. 1. Omentin inhibits TNF-α induced expression of ICAM-1 and VCAM-1 in a dose-dependent manner in HUVECs. Cells were treated with indicated concentrations of omentin for 1 h and then stimulated with TNF-a (10 ng/ml) for 6 h. (A) The effect of omentin on ICAM-1 mRNA expression. (B) The effect of omentin on VCAM-1 mRNA expression. (C) The effect of omentin on ICAM-1 protein expression. (D) The effect of omentin on VCAM-1 protein expression. Data represent the mean ± SEM ($N = 3$), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control. Omentin dose-dependently inhibits TNF-α-induced ICAM-1 and VCAM-1 expressions in HUVECs.

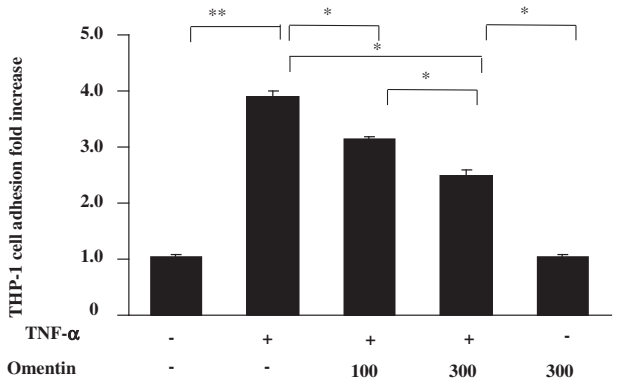

Fig. 2. Effect of omentin on adhesion of monocytes to HUVECs. Cells were incubated with various concentrations of omentin for 1 h prior to the treatment of TNF-α. THP-1 cells' adhesion to HUVECs was quantified by monocyte adhesion assay. Data represent the mean ± SEM ($N = 3$), $^{*}P < 0.05$, $^{**}P < 0.01$ versus control. Omentin suppresses adhesion of THP-1 cells to TNF-α-activated HUVECs.

enhanced chemiluminescent method, and immunoreactive bands were quantified using an imaging system (Las-4000, Fu Ji Film, Japan). The anti-IκB, anti-phospho-ERK, anti-ERK, anti-ICAM and anti-VCAM antibodies were obtained from Santa Cruz Biotechnology. Values were corrected with the absorbency of the internal control (actin).

2.6. NF-κB activity assay

NF-κB activity was determined in a whole cell extract using the ELISA-based kit (Active Motif, USA). HUVECs were washed with ice-cold PBS, scraped into tubes and centrifuged. The pellet was lysed in the Complete Lysis Buffer containing dithiothreitol and a protease inhibitor cocktail. After centrifugation at 14,000 rpm at 4 °C for 20 min, the protein concentration in the supernatant was measured by using a protein assay (bicinchoninic acid, BCA; Pierce, Rockford, IL, USA). NF-κB activity was then measured by ELISA according to manustructure's instructions. Briefly, the resulting supernants were added to a 96-well plate on which an oligonucleotide containing the NF-κB consensus binding site (5′-GGGACTTTCC-3′) has been immobilized. The active form of NF-κB contained in the cell extract specifically bound to this oligonucleotide. The primary antibody against the active form of NF-κB was added. The antibody recognizes an epitope on p65 that is accessible only when NF-κB is activated and bound to its target DNA. The experiment was performed in duplicates and results are expressed as OD450 nm.

2.7. ERK activity assay

ERK activity was determined using the chemistry-based kit (Genmed Scientifics Inc., USA), according to the manufacturer's

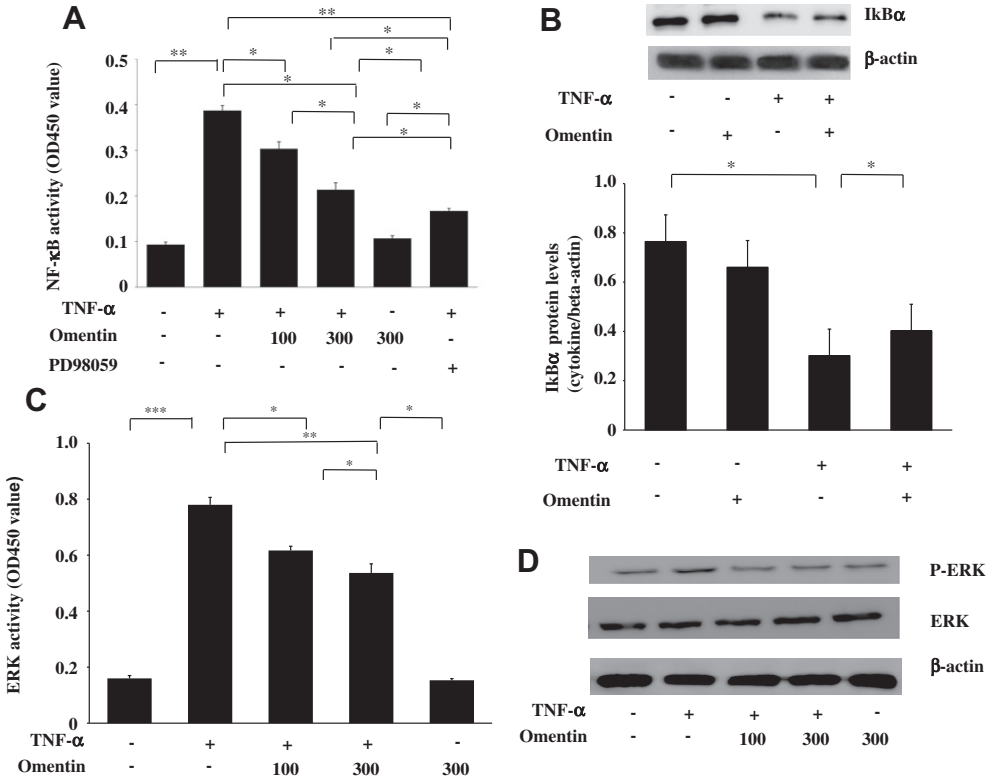

Fig. 3. Effect of omentin on NF-κB signaling in HUVECs. (A) The effects of omentin and ERK inhibitor PD98059 on NF-κB activation. HUVECs were preincubated with indicated concentrations of omentin or PD98059 for 1 h prior to incubation without or with TNF-α (10 ng/ml) for 6 h. Next, a whole cell extract was obtained and NF-κB activation was measured with the ELISA-based kit. Representative blots from more than three independent experiments are shown. (B) The effect of omentin on degradation of IkBα. Cells were pretreated with various concentrations of omentin for 1 h then TNF-α for 6 h. Their cytoplasmic portions were extracted, and protein levels of IkBa were measured on western blots. (C) The effect of omentin on ERK activation. HUVECs were preincubated with indicated concentrations of omentin for 1 h prior to incubation without or with TNF-α (10 ng/ml) for 6 h. Next, a whole cell extract was obtained and ERK activation was measured with the chemistry-based kit. Representative blots from more than 3 independent experiments are shown. (D) The effect of omentin on ERK phosphorylation. HUVECs were preincubated with indicated concentrations of omentin for 1 h prior to incubation without or with TNF-α (10 ng/ml) for 6 h. Phospho-ERK expression was measured by an anti-phospho-ERK antibody on a Western blot. Representativeblots from more than three independent experiments are shown. Data represent the mean ± SEM ($N = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ versus control. Omentin inhibits TNF-α-activated ERK/NF-κB signaling pathway in HUVECs.

instructions. Each experimental condition was tested in three different wells and measured in duplicate.

2.8. Data analysis

All quantitative variables are presented as means ± SEM from three separate experiments. SPSS 10.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis. Statistical significance was assessed by performing unpaired Student' t test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Omentin inhibits TNF-α induced expression of ICAM-1 and VCAM-1 in a dose-dependent manner in HUVECs

We first investigated whether omentin had effects on gene expression of adhesion molecules in HUVECs. Our data have shown TNF-α strongly increased ICAM-1 and VCAM-1 mRNA levels (Fig. 1A and B). When HUVECs were preincubated with omentin and then exposed to TNF-α, both ICAM-1 and VCAM-1 mRNA expression were significantly suppressed in a dose-dependent manner compared with TNF-α treatment alone. On the other hand, HUVECs with only omentin (300 ng/ml) treatment showed no significant difference in the level of ICAM-1and VCAM-1 mRNA expression compared with the untreated control.

Our results also showed that omentin pretreatment clearly inhibited ICAM-1 and VCAM-1 protein expression activated by TNF-α in a dose-dependent manner. Omentin did not lead to any significant changes in the protein expression of these adhesion molecules in the absence of TNF-α (Fig. 1C and D). All indicated omentin suppressed mRNA and protein expression of adhesion molecules by TNF-α.

3.2. Omentin suppresses adhesion of THP-1 cells to TNF-α-activated HUVECs

To investigate whether omentin could decrease THP-1 cells' adhesion to HUVECs, we examined the adherence of THP-1 cells to TNF-α-activated HUVECs with or without omentin pretreatment. Omentin pretreatment reduced TNF-α induction of THP-1 cells' adhesion by 20% at 100 ng/ml and by 37% at 300 ng/ml, respectively (Fig. 2). These results indicate that omentin inhibits TNF-α-induced adhesion of monocytes to HUVECs.

3.3. Effect of omentin on NF-κB signaling in HUVECs

HUVECs treated with TNF-α showed significant increases in NF-κB activity, whereas this induction was decreased by omentin pretreatment in a dose-dependent manner (Fig. 3A). In addition, pretreatment with omentin diminished TNF-α induced degradation of IκBα (Fig. 3B). Omentin treatment alone had no change in IkBa protein expression and NF-κB activity compared with control. These results indicate that omentin inhibits TNF-α-induced degradation of IkBα and NF-κB activation in HUVECs.

We next investigated the upstream signaling pathway for the TNF-α induction of NF-κB. We found that omentin pretreatment significantly inhibited the TNF-α-induced ERK activity in HUVECs (Fig. 3C). We also found that TNF-α directly phosphorylated ERK and that omentin decreased phosphorylation of ERK (Fig. 3D). In

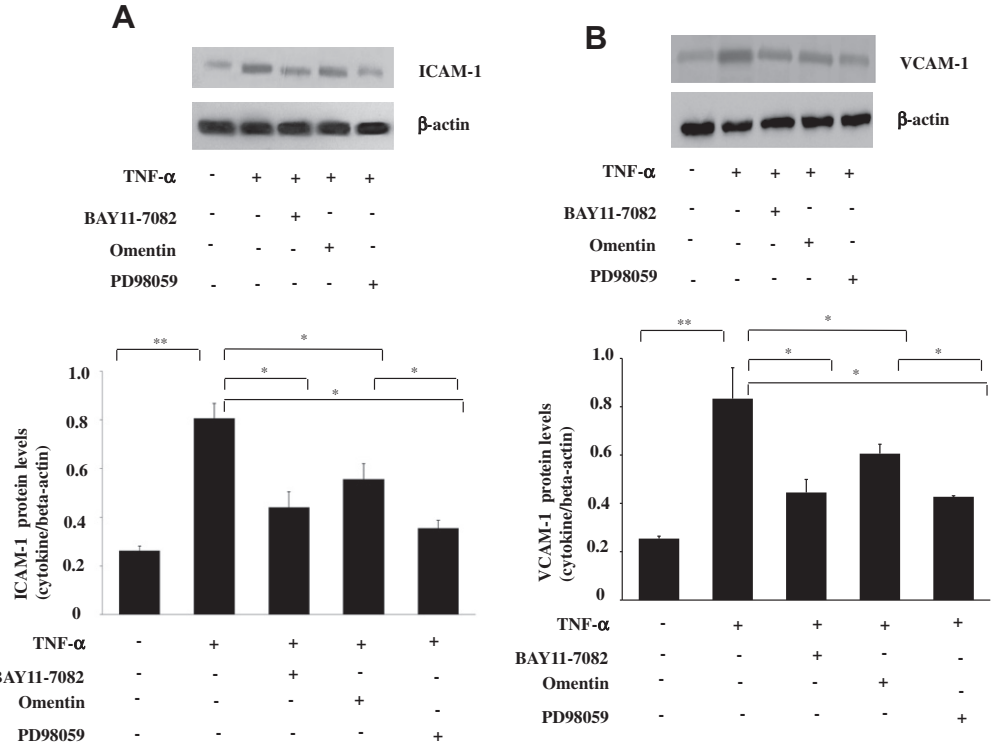

Fig. 4. Omentin and signaling inhibitors decrease TNF-α-induced ICAM-1 and VCAM-1 protein expression in HUVECs. After treatment with omentin (300 ng/ml), or signaling inhibitors (NF-κB inhibitor BAY11-7082, 50 mM; ERK inhibitor PD98059, 50 μM) for 1 h, cells were stimulated with TNF-α (10 ng/ml) for another 6 h. Total cell lysates were separated by SDS-PAGE and immunoblots were analyzed with anti-ICAM-1, anti-VCAM-1 and anti-actin antibodies. Actin was measured as an internal control. (A) The effects of omentin and signaling inhibitors on ICAM-1 protein expression. (B) The effects of omentin and signaling inhibitors on VCAM-1 protein expression. Representative blots from more than three independent experiments are shown. Data represent the mean ± SEM ($N = 3$).*$P < 0.05$ versus control. The ICAM-1 and VCAM-1 protein expression induced by TNF-α, was down-regulated by inhibition of NF-κB and ERK activity. What's more, omentin inhibits TNF-α-induced expression of VCAM-1 and ICAM-1 by blocking activation of ERK and NF-κB.

order to determine whether ERK mediates TNF-α induction of NF-κB, we investigated the effect of ERK inhibitor, PD98059 on NF-κB activity. Our data showed that pretreatment with PD98059 significantly inhibited the TNF-α-induced NF-κB activity (Fig. 3A).

3.4. Omentin and signaling inhibitors reduce ICAM-1 and VCAM-1 protein expression induced by TNF-α in HUVECs

After studying omentin inhibition of TNF-α-activated ERK/NF-κB signaling pathways, we further explored the inhibitory effect of omentin on ICAM-1 and VCAM-1 protein expression compared with NF-κB inhibitor BAY11-7082 and ERK inhibitor PD98059 in HUVECs. Increased ICAM-1 and VCAM-1 protein expression by TNF-α induction was decreased by the pretreatments with omentin, BAY11-7082 or PD98059 (Fig. 4A and B). These results indicate that TNF-α induction of ICAM-1 and VCAM-1 protein expression is regulated by NF-κb and ERK pathway. What's more, omentin inhibits TNF-α-induced expression of VCAM-1 and ICAM-1 by blocking activation of ERK and NF-κB.

4. Discussion

The present study demonstrated that omentin suppressed TNF-α-induced ICAM-1 and VCAM-1 expression in endothelial cells, leading to the suppression of THP-1 cells adhesion to HUVECs. Our results also indicated that omentin inhibition of TNF-α-activated ERK/NF-κB pathway contributed to the suppression of ICAM-1 and VCAM-1 expression at least in part.

ICAM-1 and VCAM-1, as reflecting endothelial dysfunction biomarkers, play key roles at the early stage of inflammatory response to facilitate leukocytes adhesion and transmigration in vascular endothelial cells [18]. The expression of adhesion molecules such as ICAM-1 and VCAM-1 on the endothelial cell surface is the initial step in atherogenesis. Circulating levels of sICAM-1 and sVCAM-1 were reported to be directly associated with obesity and other coronary heart disease risk factors and might serve as predictors of coronary heart disease [19,20]. Adipokines such as visfatin, adiponectin and resistin regulate the endothelial expression of cell adhesion molecules [15,21–23]. As expected, we found a significant increase in ICAM-1 and VCAM-1expression at the mRNA and protein levels in TNF-α treated HUVECs. Futherly, we observed a significant reduction in ICAM-1 and VCAM-1 mRNA and protein levels, when TNF-α-stimulated HUVECs were pre-treated with omentin. Our results also indicate that omentin inhibits TNF-α-induced adhesion of monocytes to HUVECs. These results suggest that omentin has anti-inflammatory action to attenuate endothelial dysfunction and atherosclerosis.

NF-κB is one of the most critical transcription factors involved in inflammation. In vascular inflammatory responses, NF-κB regulates endothelial adhesion molecules and chemokines which contribute to atherosclerosis and atherothrombosis [24]. Activation of ERK has been shown to be involved in various vessel diseases, such as atherosclerosis [25]. In this study, omentin observably inhibits TNF-α-induced degradation of IκBα, NF-κB activation and ERK activation in HUVECs. Further results showed that omentin, NF-κB inhibitor BAY11-7082 and ERK inhibitor PD98059 reduce ICAM-1 and VCAM-1 protein expression induced by TNF-α in endothelial cells. In addition, pretreatment with PD98059 significantly inhibited the TNF-α-induced NF-κB activity in HUVECs. These results suggest that omentin inhibition of TNF-α-induced expression of VCAM-1 and ICAM-1 is mediated by its down-regulation of ERK/NF-κB signaling pathway.

Our previous study have demonstrated that serum concentrations of omentin-1 may be related to CAD [10]. Omentin is also considered to be associated with carotid atherosclerosis in patients with metabolic syndrome [26] and with carotid artery intima-media thickness in apparently healthy men [27]. Yamawaki et al. [28] recently indicate that Omentin inhibits COX-2 induction via preventing the JNK activation presumably through activation of AMPK/eNOS/NO pathways, contributing to suppress TNF-induced vascular inflammation in human endothelial cells. In our present study, omentin inhibited TNF-α-induced expression of adhesion molecules in endothelial cells via blocking ERK/NF-κB pathway. The evidence of activated NF-κB and increased ICAM-1 and VCAM-1 expression in human atherosclerotic plaques has suggested a very important role for NF-κB-mediated ICAM-1 and VCAM-1 expression in the early stage of atherosclerosis [29,30]. Taken together, these findings may provide insights into the mechanism of omentin with respect to anti-atherosclerotic activity.

In conclusion, our results demonstrate that omentin inhibits ICAM-1 and VCAM-1 expression via interruption of ERK/NF-κB signaling pathway and subsequently suppresses adhesion of monocytes to TNF-α-activated endothelial cells. Our findings may provide a potential role for omentin in the pathogenesis of endothelial dysfunction and atherosclerosis. And it may contribute to the development of new therapies for obesity-related diseases. However, potential involvement of other signaling pathways and the precise underlying mechanisms still need further investigation.

Acknowledgments

We would like to express gratitude to Ms. Cui Bin for her continuous support of our investigations. This work was supported by promotive research fund for excellent young and middle-aged scientists of Shandong Province (BS2011044 and BS2010YY053).

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