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ORIGINAL ARTICLE

Omentin inhibits osteoblastic differentiation of calcifying vascular smooth muscle cells through the PI3K/Akt pathway

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Abstract Arterial calcification is positively associated with visceral adiposity, but the mechanisms remain unclear. Omentin is a novel adipokine that is selectively expressed in visceral adipose tissue. The levels of circulating omentin are decreased in obesity, and they correlate negatively with waist circumference. This study investigated the effects of omentin on the osteoblastic differentiation of calcifying vascular smooth muscle cells (CVSMCs), a subpopulation of aortic smooth muscle cells putatively involved in vascular calcification. Omentin inhibited mRNA expression of alkaline phosphatase (ALP) and osteocalcin; omentin also suppressed ALP activity, osteocalcin protein production, and the matrix mineralization. Furthermore, omentin selectively activated phosphatidylinositol 3-kinase (PI3K) downstream effector Akt. Moreover, inhibition of PI3K or Akt activation reversed the effects of omentin on ALP activity and the matrix mineralization. The present results demonstrate for the first time that omentin can inhibit osteoblastic differentiation of CVSMCs via PI3K/Akt signaling pathway, suggesting that the lower omentin levels in obese (specially visceral obese) subjects contribute to the development of arterial calcification, and omentin plays a protective role against arterial calcification.

Keywords Omentin · Vascular smooth muscle cells · Calcification · Phosphatidylinositol 3-kinase

Introduction

Adipose tissue is now regarded as a highly metabolic active endocrine organ that, in addition to regulating fat mass and nutrient homeostasis, releases a large number of bioactive mediators (adipokines) modulating energy homostasis, blood pressure, glucose and lipid metabolism, neuroendocrine function, cell viability, feeding, reproduction, inflammation, and, importantly, cardiovascular function (Trujillo and Scherer 2006; Fantuzzi and Mazzone 2007; Gualillo et al. 2007).

Omentin is a novel adipokine codified by two genes (1 and 2) that is selectively expressed in visceral adipose tissue and is highly abundant in plasma (Kralisch et al. 2005; Schaffler et al. 2005; Yang et al. 2006). The concentration of omentin-1, the major circulating isoform in human plasma, was determined to be 100 ng to 1 μg/ml (Yang et al. 2006). The levels of circulating omentin-1 are decreased in obesity, and they correlate with several markers of the metabolic syndrome, positively with adiponectin and high-density lipoprotein (HDL) levels and negatively with body mass index (BMI), waist circumference, insulin resistance and leptin level (de Souza Batista et al. 2007; Moreno-Navarrete et al. 2010). Therefore, the study of the possible regulatory role of

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omentin in cardiovascular disease pathogenesis will be an intriguing tool for further research in this field.

Vascular calcification has severe clinical consequences in a number of diseases including atherosclerosis, diabetes, and end-stage renal failure, and it is now considered an independent prognostic indicator of future adverse cardiovascular events (Abedin et al. 2004; Johnson et al. 2006; Guzman 2007; Demer and Tintut 2008). Moreover, vascular calcification is now recognized as highly regulated process which is similar in many ways to bone mineralization. During vascular calcification, several vascular cell types, such as vascular smooth muscle cells (VSMCs), pericytes, and fibroblasts, are transformed into osteoblast-like phenotypes in which VSMCs are implicated primarily (Abedin et al. 2004; Johnson et al. 2006; Guzman 2007; Demer and Tintut 2008). In vitro, these transformed VSMCs express bone-associated genes and proteins, such as alkaline phosphatases (ALP) and osteocalcin, and are able to produce mineralized nodules.

Recent data supported that phosphatidylinositol 3-kinase (PI3K)/Akt has a crucial role in the prevention of VSMC calcification (Radcliff et al. 2005; Collett et al. 2007; Son et al. 2007). Furthermore, in vitro experiments showed that treatment with recombinant omentin enhances Akt phosphorylation in adipocytes (Yang et al. 2006).

The purpose of this study was to test the effect of omentin on osteoblastic differentiation of calcifying vascular smooth muscle cells (CVSMCs) and to identify the signaling pathway involved.

Materials and methods

Reagents

Recombinant human omentin was purchased from Cell Science Inc. (Canton, MA, USA). LY294002 and 1L-6-hydroxymethyl-chiro-inositol 2-(*R*)-2-omethyl-3-o-octadecylcarbonate (HIMO) were purchased from Calbiochem Corp. (San Diego, CA, USA). Antibodies for phosphorylated ERK1/2 (p-ERK1/2), p-p38, p-JNK, p-Akt, ERK1/2, p38, JNK and Akt were purchased from Cell Signaling Inc. (Danvers, MA, USA). Anti-mouse and anti-rabbit IgG horseradish peroxidase (HRP)-conjugate antibodies were purchase from Santa Cruz Biotechnology Inc.

Cell culture

Small sections of thoracic aorta were obtained from human cardiac transplant donor. CVSMCs, the subpopulation of aortic smooth muscle cells, were isolated and identified as described previously (Bostrom et al. 1993; Watson et al. 1994). The approval was granted by the central south university ethics review board. CVSMCs were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) containing 15% fetal bovine serum (FBS; Gibco-BRL Corp., Grand Island, NY, USA) and supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml), all from Sigma. From 6 days before Alizarin Red S staining, 5 mM β-glycerophosphate and 4 mM $CaCl_2$ were added to the media to permit maximal mineralization.

Real-time quantitative RT-PCR assay for mRNA expression

Real-time quantitative RT-PCR analysis was done using Roche Molecular LightCycler (Roche Applied Science, Indianapolis, IN, USA) as described previously (Xie et al. 2007a, 2010a, b), which is a combined thermal cycler and fluorescence detector with the ability to monitor the progress of individual PCR reactions optically during amplification. Total RNA from cultured cells was isolated using Trizol reagent (Gibco), and reverse transcription was performed using 1.0 μg total RNA and the reverse transcription system (Promega). Amplification reactions were set up in 25 μl reaction volumes containing amplification primers and SYBR Green PCR Master Mix (PE Applied Biosystems). A 1 μl volume of cDNA was used in each amplification reaction. Preliminary experiments were carried out for primer concentration optimization. The PCR primers were as follows: ALP sense, 5′-GACCTCCTCGGAAGACACTC-3′; ALP antisense, 5′-AGGCCCATTGCCATACAG-3′; osteocalcin sense, 5′-GCAGAGTCCAGCAAAGGTG-3′; osteocalcin antisense, 5′-GCTCCCAGCCATTGATACAG-3′; β-actin sense, 5′-CCCAGCCATGTACGTTGCTA-3′, β-actin antisense, 5′-AGGGCATACCCCTCGTAGATG-3′.

Amplifications were performed, and calibration curves were run in parallel in triplicates for each analysis. Each sample was analyzed three times during each experiment. The experiments were carried out at least twice. Amplification data were analyzed using the Sequence Detector System Software (PE Applied Biosystems). Relative quantification were calculated by normalizing the test crossing thresholds (Ct) with the β-actin amplified control Ct. The results were normalized to β-actin and expressed as percentage of controls.

ALP activity, osteocalcin secretion assay

CVSMCs were grown to confluence in 24-well plates. The cells were washed three times with PBS, and the monolayer was scraped into lysis solution containing 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.02% NaN_3, and 1 μg/mL aprotinin. The lysates were homogenized by sonication for 20 s. ALP activity was then

measured in the samples derived from each culture using an ALP Kit (Sigma) as described previously (Xie et al. 2006; Tang et al. 2007). Osteocalcin released into the culture media was measured using a specific radioimmunoassay kit (DiaSorin Corp., Stillwater, MN) as described previously (Xie et al. 2006; Tang et al. 2007). To normalize protein expression to total cellular protein, a Bradford assay was used to quantify protein from the lysate solution.

Measurement of matrix mineralization

CVSMCs in 24-well plates were cultured in the presence of vehicle or other agents for 18 days. Then, the extent of matrix mineralization was determined by Alizarin Red S staining (Bodine et al. 1996; Li et al. 2009). Briefly, cells were fixed in 70% ethanol for 1 h at room temperature and stained with 40 mM Alizarin Red S for 10 min. Next, cell preparations were washed with PBS to eliminate nonspecific staining. As described by Bodine et al. (1996), Alizarin Red S staining was released from cell matrix by incubation in cetyl-pyridinium chloride for 15 min. The amount of released dye was quantified by spectrophotometry at 540 nm. Results were then normalized to total cellular protein values.

Detection MAPK and Akt activation by Western blot analysis

Western blot analysis was performed as described previously (Xie et al. 2007b, 2008, 2010a, b; Liao et al. 2010). Treated cells were washed quickly with cold PBS containing 5 mM EDTA and 0.1 mM Na_3VO_4, and lysed with a lysis buffer consisting of 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10 mM NaH_2PO_4, 10% glycerol, 2 mM Na_3VO_4, 10 mM NaF, 1 mM ABSF, 10 μg/ml leupeptin and 10 μg/ml aprotinin. The protein concentrations were determined using a Bradford protein assay. Equal amount of protein (50 μg/lane) was loaded onto a 7.5% polyacrylamide gel. After electrophoresis, the SDS-PAGE separated proteins were transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was blocked with 2.5% nonfat milk in PBS and then incubated with the p-ERK1/2, ERK1/2, p-p38, p38, p-JNK, JNK, p-Akt and Akt primary antibodies which were then detected with the appropriate HRP-conjugated secondary antibodies. Blots were processed using an ECL Kit (Santa Cruz) and exposed to X-ray film.

Measurement of cAMP level

CVSMCs were cultured in 24-well plate. After 30 min incubation with vehicle or omentin (1,000 ng/ml) or TNF-α (100 ng/ml), the medium was aspirated. cAMP concentration was measured after trichloracetic acid precipitation of the cell extracts using a cAMP assay kit (China Atomic Energy Institute, PR China).

Statistical analysis

SPSS 11.0 was used for the statistical analyses. The results were provided as mean ± SD. Comparisons were made using a one-way ANOVA. All in vitro experiments were repeated at least three times, and representative experiments were shown.

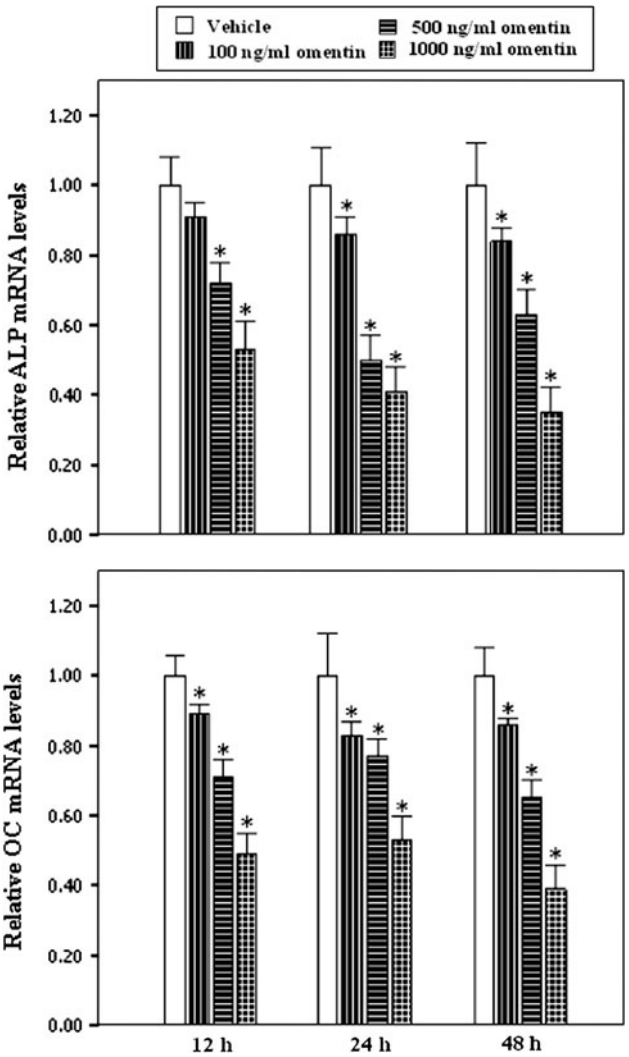

Fig. 1 Effect of omentin on ALP and osteocalcin mRNA expression in CVSMCs. RNA was isolated from cells treated with vehicle and 100, 500 and 1,000 ng/ml omentin for 12, 24 or 48 h. Expression was assessed by real-time quantitative RT-PCR and normalized to *β*-actin. Results are expressed as percents of control cells. The *bars* represent the mean ± SD ($n = 3$; $^*P < 0.05$ versus vehicle-treated control cells)

Results

Effect of omentin on osteoblastic differentiation of CVSMCs

Quantitative real-time RT-PCR analysis showed that treatment of cells with 100, 500, or 1,000 ng/ml omentin for 12, 24 or 48 h dose-dependently inhibited the transcription of ALP and osteocalcin in CVSMCs (Fig. 1).

Treatment with 100–1,000 ng/ml omentin dose-dependently inhibited ALP activity and osteocalcin production in CVSMCs (Fig. 2a, b). After 12–48 h in culture with 1,000 ng/ml omentin, the ALP activity and osteocalcin production decreased significantly in a time-dependent manner in CVSMCs (Fig. 2a, b).

Furthermore, 18 days of treatment with 100–1,000 ng/ml omentin dose-dependently suppressed the matrix mineralization in CVSMCs (Fig. 3). Figure 3a showed the representative entire plate views of the Alizarin Red S staining. We observed a significant 22, 41 and 85% reduction in Alizarin Red S staining in 100, 500 and 1,000 ng/ml omentin treated cells compared with control cells, respectively (Fig. 3b).

Effect of omentin on Akt activation

Cells were treated with 100 ng/ml TNF-α (a compound that could induce ERK, p38 and JNK activation in human VSMCs (Rajesh et al. 2008; Suh et al. 2006, 2009) was used as a positive control) for 15 min or 1,000 ng/ml omentin for 5–60 min.

Western blot analysis showed that omentin stimulated the activity of Akt in CVSMCs after 5 min of incubation, as demonstrated by an increase in the levels of phosphorylated Akt, slightly at 5 min, and more significantly at 30 and 60 min; the peak activation of Akt occurred at 30 min (Fig. 4a). In contrast, omentin could not activate ERK, p38 and JNK (Fig. 4b). However, TNF-α-treated cells showed p-ERK, p-p38 and p-JNK bands (Fig. 4b). Figure 4c showed that the activation of Akt by omentin was inhibited by the PI3K inhibitor LY294002 or Akt inhibitor HIMO.

Effect of omentin on intracellular cAMP production

Cells were treated with vehicle or 100 ng/ml TNF-α (a compound that could increase intracellular cAMP level

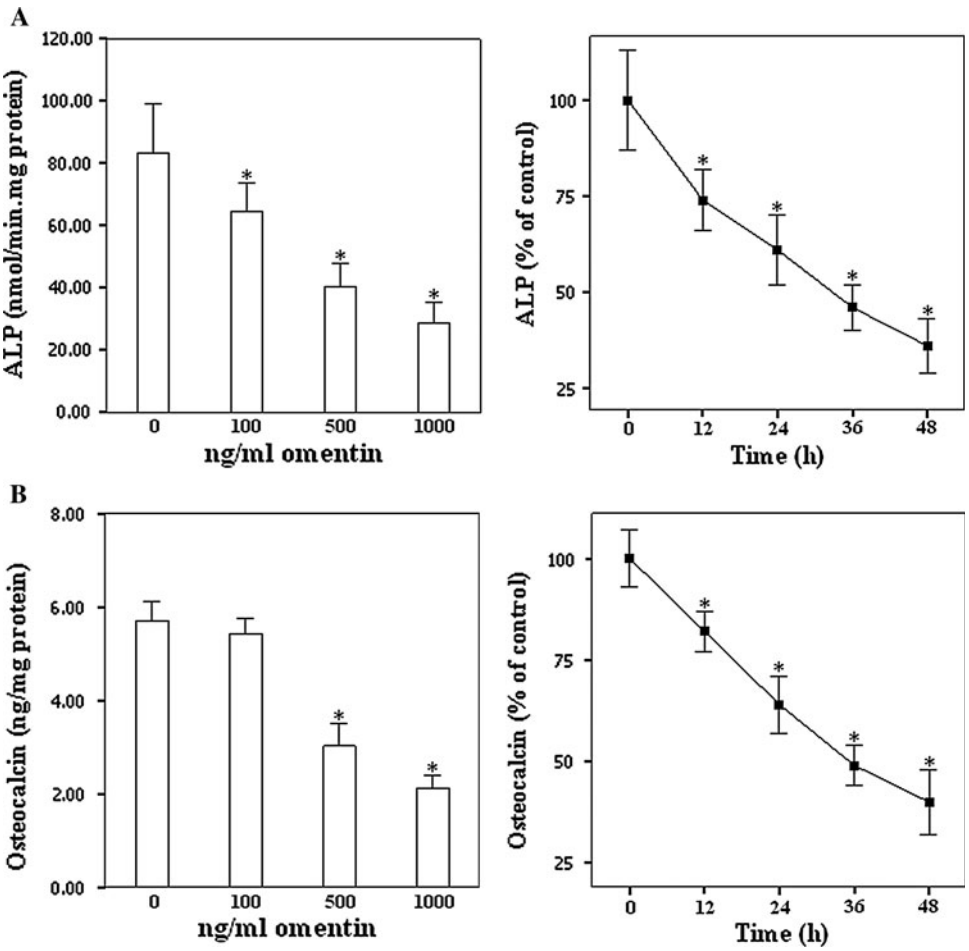

Fig. 2 Effects of omentin on ALP activity and osteocalcin secretion in VSMCs. Cells were exposed to 100–1,000 ng/ml omentin for 48 h and to 1,000 ng/ml omentin for 12–48 h. The cells were homogenized for ALP activity assay. Cell culture media was collected for osteocalcin secretion assay. **a** The dose and time response of omentin on ALP activity in cultured CVSMCs. *Bar* represents mean ± SD ($n = 6$; $^*P < 0.05$ versus control). *Dots* represent the percent of control ALP activity level at various time-points ($n = 6$; $^*P < 0.05$ versus control). **b** The dose and time response of omentin on osteocalcin secretion in cultured CVSMCs. Bar represents mean ± SD ($n = 6$; $^*P < 0.05$ versus control). *Dots* represent percent of control osteocalcin secretion level at various time-points ($n = 6$; $^*P < 0.05$ versus control)

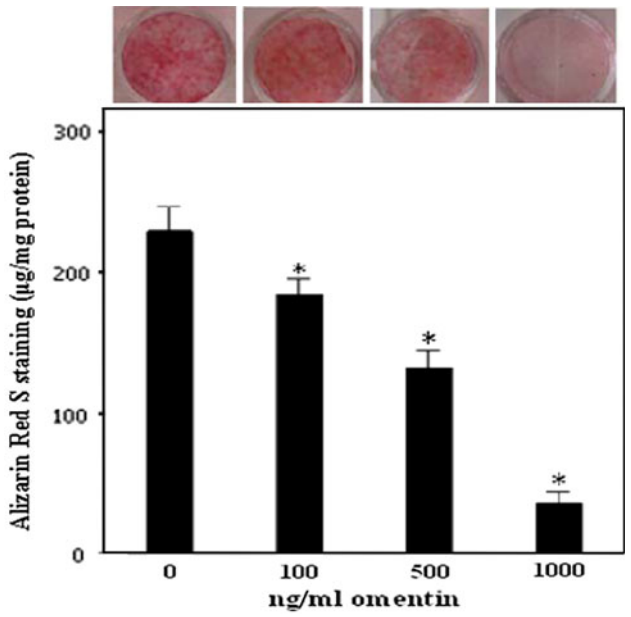

Fig. 3 Omentin inhibited the matrix mineralization in CVSMCs. CVSMCs in 24-well plates were cultured in DMEM containing 10% FBS, in the presence of 100, 500 or 1,000 ng/ml omentin or vehicle for 18 days. Matrix mineralization was determined by Alizarin Red S staining. *Upper panel*: the representative entire plate views of the Alizarin Red S staining in 24-well plates. *Lower panel*: quantification of Alizarin Red S staining via extraction with cetyl-pyridinium chloride. The amount of released dye was quantified by spectrophotometry at 540 nm ($n = 4$; $^*P < 0.05$ versus control)

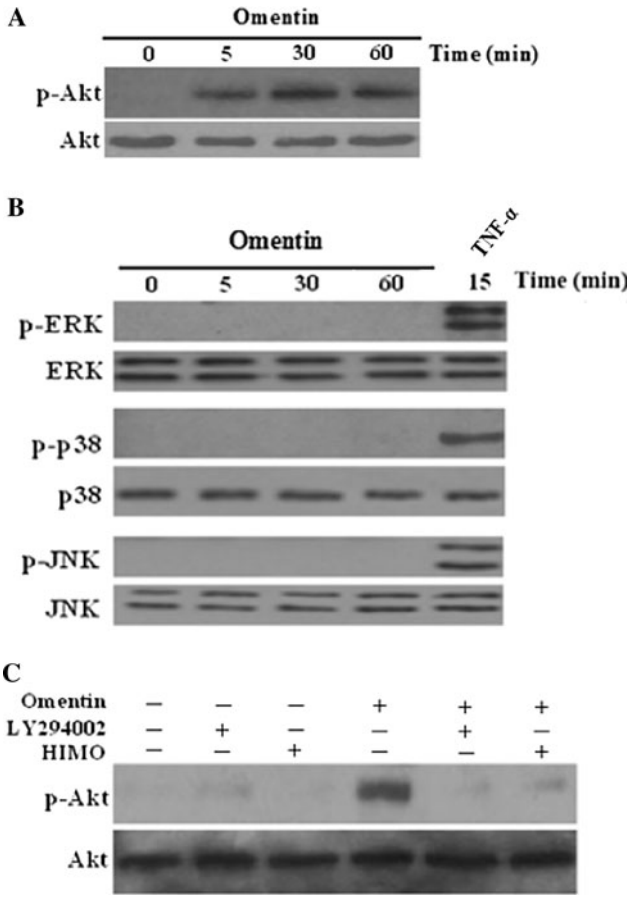

Fig. 4 Effects of omentin on ERK, p38, JNK and Akt activation in CVSMCs. Cell lysates were subjected to Western blot and incubated with ERK, p38, JNK, Akt and their phosphorylated antibodies. Shown are representative results for (**a**, **b**) cells exposed to 1,000 ng/ml omentin for 5–60 min or 100 ng/ml TNF-α for 15 min; **c** cells incubated with LY294002 (10 μM) or HIMO (10 μM) for 1 h prior to treatment with 1,000 ng/ml omentin for 30 min

in CVSMCs (Tintut et al. 2000) was used as a positive control) or 100–1,000 ng/ml omentin for 30 min.

Figure 5 shows that omentin has no effect on intracellular cAMP production in CVSMCs. As expected, TNF-α-treated cells showed increased intracellular cAMP level.

Effect of PI3K/Akt signaling pathway on the anti-osteogenic action of omentin

Treatment of cells with LY294002 or HIMO abolished the inhibitory effects of omentin on ALP activity (Fig. 6a) and the matrix mineralization (Fig. 6b), suggesting that omentin inhibits osteoblastic differentiation of CVSMCs via PI3K/Akt signaling pathway.

Discussion

Omentin is a secretory protein that has been recently identified as a new adipokine. Omentin is highly expressed in the stroma vascular fraction of visceral fat and is highly abundant in plasma (Kralisch et al. 2005; Schaffler et al. 2005; Yang et al. 2006). However, there were no data on the effect of omentin on VSMCs. The present results indicate that physiological concentrations (100–1,000 ng/ml) of omentin inhibit osteoblastic differentiation of CVSMCs. The inhibitory effects of omentin on calcification of CVSMCs appear to be through the enhancement of PI3K/Akt signaling.

Obesity is a heterogeneous condition with respect to regional distribution of fat tissue; visceral (central) obesity refers to fat accumulation within omental and mesenteric fat depots, whereas peripheral obesity generally refers to subcutaneous fat accumulation. Evidence from a number of sources (Goodpaster et al. 2003; Nicklas et al. 2003, 2004; Wagenknecht et al. 2003; Sironi et al. 2004; Fox et al. 2007) has shown that visceral obesity is associated with a higher risk of cardiovascular disease and its risk factors, including diabetes mellitus, insulin resistance, hypertension, and dyslipidemia, than is peripheral obesity.

Cardiovascular complications of obesity have always been well known, but the intensive research on adipokines that evolved in the last two decades has finally provided a plausible link between obesity (especially visceral obesity)

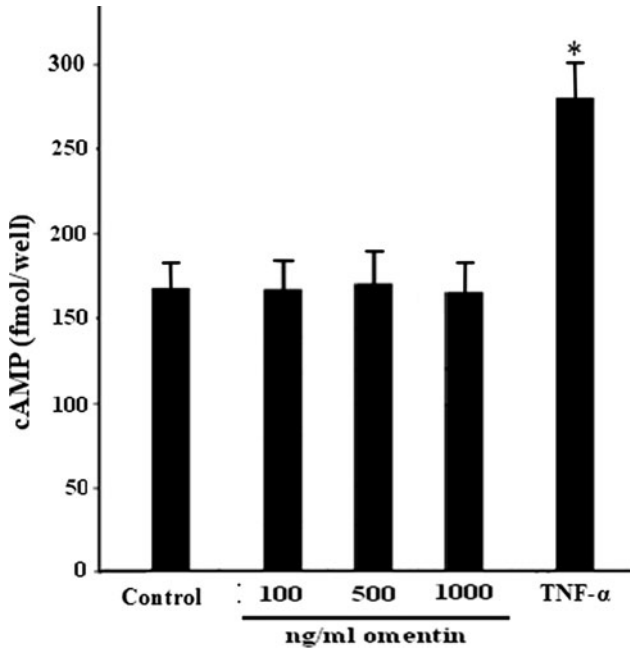

Fig. 5 Effect of omentin on intracellular cAMP level in CVSMCs. Cells were cultured in 24-well culture plate and incubated with the vehicle or omentin (100–1,000 ng/ml) or TNF-α (100 ng/ml) for 30 min. cAMP level in cell lysates was determined using a cAMP assay kit. The *bars* represent the mean ± SD ($n = 4$; $^*P < 0.05$ versus control)

and the cardiovascular diseases (Després and Lemieux 2006). Since adipokines derived from omental adipose tissue are clearly involved in cardiovascular disease (Kralisch et al. 2005; Trujillo and Scherer 2006; Fantuzzi and Mazzone 2007; Gualillo et al. 2007), new omental adipokines such as omentin might play an important role in the pathogenesis of cardiovascular disease.

Both plasma omentin and adipose tissue omentin mRNA levels are inversely related to obesity. Plasma omentin correlates negatively with BMI, waist circumference, leptin, fasting insulin and insulin resistance and positively with HDL (de Souza Batista et al. 2007; Moreno-Navarrete et al. 2010). It is interesting to note that this pattern of results for omentin is similar to adiponectin, an insulin sensitizer and cardiovascular-protective adipokine (Matsuzawa 2005; Bełtowski et al. 2008; Guerre-Millo 2008). In fact, circulating adiponectin levels were positively correlated with omentin values (de Souza Batista et al. 2007). This may be important since adiponectin and omentin are the only two adipokines reported to date whose levels in adipose tissue decrease in human obesity (de Souza Batista et al. 2007; Guerre-Millo 2008; Moreno-Navarrete et al. 2010). The inverse relationship between obesity and both omentin and adiponectin may suggest that higher omentin or adiponectin levels may be similarly seen as markers for leanness or as positive factors that oppose the obese state and its pathophysiological consequences.

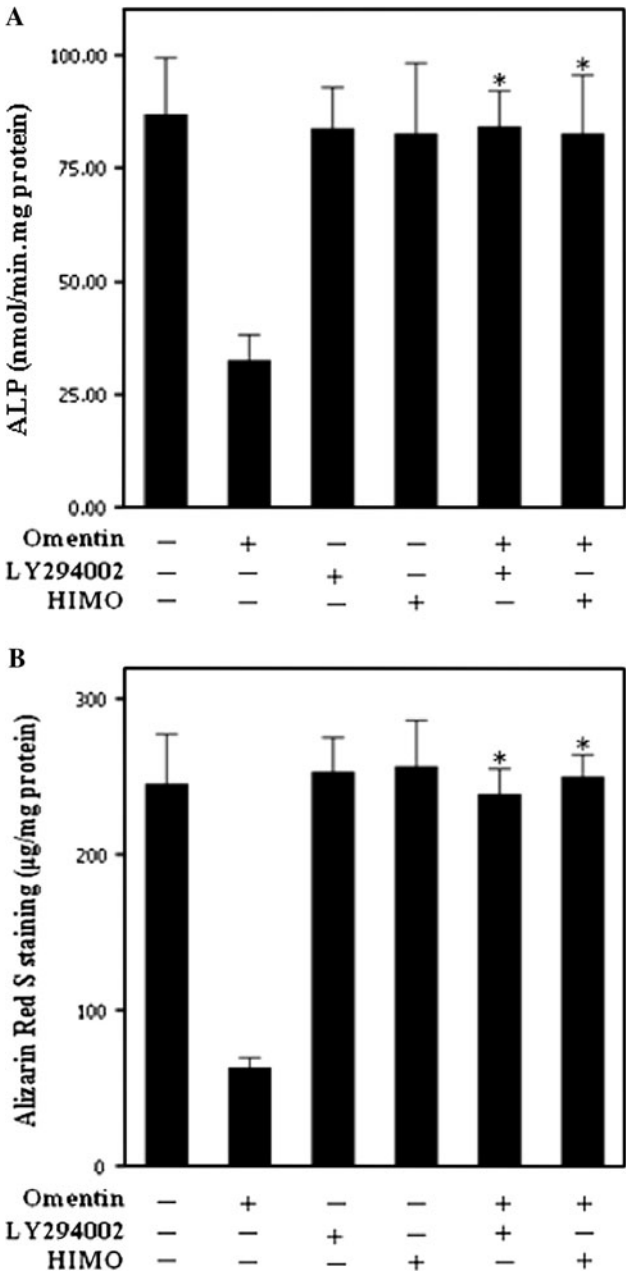

Fig. 6 Effect of PI3K/Akt inhibition on inhibitory action of omentin on osteoblastic differentiation in CVSMCs. **a** CVSMCs were pre-treated with vehicle or LY294002 (10 μM) or HIMO (10 μM) for 3 h prior of treatment with 1,000 ng/ml omentin for 48 h. The *bars* represent the mean ± SD ($n = 6$; $^*P < 0.05$ versus omentin-treated control). **b** Vehicle, LY294002 (10 μM), HIMO (10 μM), omentin (1,000 ng/ml), LY294002/omentin or HIMO/omentin were added to the cells and cultures were continued for 16 days, the culture media were changed every 3 days in this culture period. The Alizarin Red S staining was performed and quantified as described in Fig. 3. The *bars* represent the mean ± SD ($n = 4$; $^*P < 0.05$ versus omentin-treated control)

Both abdominal adiposity and arterial calcification are related to cardiovascular events (Wilson et al. 2001; Bigaard et al. 2004; Janssen et al. 2004; Vliegenthart et al.

2005), indicating that the two could be linked. Several studies have reported positive correlations between visceral abdominal fat and artery calcification (Arad et al. 2001; Allison and Michael Wright 2004; Conway et al. 2007; Lee et al. 2007; Golledge et al. 2008). Arad et al. (2001) reported that coronary artery calcium (CAC) scores were positively correlated with intra-abdominal adiposity. A cross-sectional analytic study revealed that central adiposity is an independent predictor of coronary artery calcification (Allison and Michael Wright 2004). Lee et al. (2007) found that abdominal obesity measured by waist girth or waist-hip ratio (WHR) is positively associated with early coronary calcification in young adults. Conway et al. (2007) demonstrated that the presence of CAC was positively associated with visceral adiposity in men and women after adjustment for age and other traditional cardiovascular risk factors. Golledge et al. (2008) identified a positive association between a surrogate measure of visceral adipose and aortic calcification. It has been known that plasma adiponectin levels were associated with progression of coronary artery calcification in type 1 diabetic and nondiabetic subjects, independent of other cardiovascular risk factors (Maahs et al. 2005). Furthermore, a recent study reported that adiponectin had an inhibitory effect on inorganic phosphate (Pi)-induced apoptosis and calcification in VSMCs (Son et al. 2008). In the present study, we firstly demonstrated that omentin can also inhibit the osteoblastic differentiation of CVSMCs.

To gain further insight into the mechanisms by which omentin inhibited osteoblastic differentiation of CVSMCs, we evaluated the signaling events. Mitogen-activated protein kinases (MAPKs) and PI3K/Akt are well known to play the essential role in controlling major cell fate decisions such as proliferation, apoptosis and differentiation (Blüthgen and Legewie 2008; Franke 2008). Recent data supported that PI3K/Akt had a crucial role in the prevention of VSMCs calcification (Radcliff et al. 2005; Collett et al. 2007; Son et al. 2007). Radcliff et al. (2005) suggested that insulin-like growth factor-I (IGF-I) inhibited osteoblastic differentiation and mineralization of VSMCs via PI3K pathway. Son et al. (2007) showed that statins inhibited Pi-induced calcification of VSMCs by up-regulation of Akt phosphorylation. Collett et al. (2007) demonstrated that the Axl receptor tyrosine kinase inhibited mineral deposition by VSMCs via activation of PI3K/Akt signaling. Furthermore, in vitro experiments showed that treatment with recombinant omentin triggered Akt signaling in adipocytes (Yang et al. 2006). The present results reveal that omentin inhibits the osteoblastic differentiation of CVSMCs via the PI3K/Akt pathway.

Because the cAMP pathway promotes osteoblastic differentiation of CVSMCs (Tintut et al. 1998), we also examined the involvement of the cAMP pathway in response to omentin. Our present data demonstrate that omentin exert no effect on cAMP production by CVSMCs. It is suggested that cAMP pathway is not involved in the regulation of osteoblastic differentiation of CVSMCs by omentin.

In conclusion, our findings demonstrated that omentin inhibited osteoblastic differentiation of CVSMCs through PI3K/Akt signaling pathway. It is suggested that the lower omentin levels in obese (specially visceral obese) subjects contribute to the development of arterial calcification, and omentin plays a protective role against arterial calcification.

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