

# Visfatin induces cholesterol accumulation in macrophages through up-regulation of scavenger receptor-A and CD36

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**Abstract** As a new potential inflammatory mediator, visfatin plays an important role in inflammation and atherosclerosis. The formation of macrophage-derived foam cells occurs at the early stage of atherosclerosis and underlies the visible fatty streak. Recent studies have indicated that visfatin may be associated with the development of foam cells, but its exact effect and molecular mechanism remain unknown. This study aims to study the effect of visfatin on foamy cell formation and its underlying molecular mechanism. Visfatin levels were determined in apolipoprotein E (ApoE) knockout (KO) mice on a western diet for 16 weeks. Effects of visfatin in cholesterol accumulation were studied both *in vivo* and *in vitro*. The levels of scavenger receptors located in macrophage surface were measured in RAW264.7 cells after treatment with visfatin. Visfatin levels were much higher in ApoE KO mice than that in the control mice. Meanwhile, oxidized low-density lipoprotein induces both

visfatin release from RAW264.7 cells and its cellular levels within 24 h. Visfatin promotes lipid accumulation mainly through excessive cholesterol uptake not only in RAW264.7 cells but also in peritoneal macrophages isolated from ApoE KO mice. Furthermore, visfatin induces the activation of scavenger receptors (SR)-A and cluster of differentiation (CD)36, but not that of SR-BI, ATP-binding cassette transporter (ABC)A1, or ABCG1 in RAW264.7 cells. Both transcriptional and posttranscriptional regulation may work in concert to mediate the expression of SR-A and CD36 in visfatin-treated cells. Visfatin induces cholesterol accumulation in macrophages and accelerates the process of atherosclerosis mainly through modulating the expression of SR-A and CD36.

**Keywords** Atherosclerosis · Visfatin · Foam cell · Scavenger receptor

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## Introduction

Atherosclerosis, the most common causes of death from coronary artery disease and cerebrovascular disease in developed countries, is characterized by chronic inflammation and redundant cholesterol accumulation within the artery wall, especially in the coronary artery and aorta (Glass and Witztum 2001; Lusis 2000). The fatty streak is the earliest visible atherosclerotic lesion and plays a key role in the development of atherosclerosis (Berliner and Heinecke 1996). The atherosclerotic plaque progression, rupture, and thrombosis always lead to acute coronary syndrome and stroke (Lusis 2000; Ross 1999; Kleemann et al. 2008). The functional disorder of arterial intima induced by kinds of

impaired factors, following the migration and differentiation of monocytes here, is a crucial step in the initiation of atherosclerosis (Lusis 2000; Wu et al. 1992; Castagna et al. 2012). The monocyte-derived macrophages swallow unwanted self-modified lipoprotein via scavenger receptors (SRs) located on its surface resulting in foamy cell formation, which underlies the visible fatty streak (Kleemann et al. 2008; Li and Glass 2002). Cholesterol accumulation in macrophage-derived foam cells is due to the broken balance between uptake and reverse transport of cholesterol, which is mainly because of uncontrolled lipid uptake and reduced cholesterol efflux (Kleemann et al. 2008; Collot-Teixeira et al. 2007; Cheng et al. 2011). There are several classes of SRs on macrophage membrane, in which class A (SR-A) and B (SR-B) attract more attention. SR-A and cluster of differentiation (CD)36 are mainly responsible for the uptake of oxidized low-density lipoprotein (ox-LDL) (Collot-Teixeira et al. 2007; Kunjathoor et al. 2002), while SR-B type I (SR-BI) mediates reverse cholesterol transport (Ji et al. 2011). Meanwhile, both ATP-binding cassette transporter A1 and G1 (ABCA1 and ABCG1) participate in the process of efflux of intracellular cholesterol to high-density lipoprotein (HDL) (Cheng et al. 2011; Chen et al. 2011). Thus, the formation of foam cells is predominantly regulated by these SRs and reverse cholesterol transporters (RCTs). Recently, a growing body of evidences has shown that some kinds of adipokine, such as adiponectin, leptin, and vaspin, may affect cholesterol accumulation in macrophages and, thus, accelerate or retard the process of atherosclerosis through modulation of SRs or RCTs (Chen et al. 2010; Spiroglou et al. 2010; Kopff and Jegier 2005; Kjerrulf et al. 2006).

Visfatin (also known as pre-B cell-enhancing factor or PBEF) is an adipokine of which early studies have mainly focused on the metabolic-associated diseases (e.g., obesity and diabetes) (Paschou et al. 2010; De Luis et al. 2008). With the discovery of pro-inflammatory role of visfatin, its potential effect in inflammation has gradually attracted much attention, especially in atherosclerosis, which is closely related with both lipid metabolism and inflammation (Yan et al. 2010; Liu et al. 2009). One of the most related studies has reported that visfatin should be considered as an inflammatory mediator, localized to foam cell macrophages within unstable atherosclerotic lesions, which potentially plays a role in plaque destabilization (Dahl et al. 2007). Although it has been reported that visfatin is participated in the pathogenesis of atherosclerosis and may be involved in the early formation of foam cells, its exact effect is largely unknown. There are still no reports whether visfatin could regulate SRs or RCTs.

The present study aimed to investigate the expression of visfatin in macrophage foam cells *in vivo* and *in vitro*. We

also studied the impact of visfatin on foamy cell formation and further explored its molecular mechanism.

## Materials and methods

### Reagents

The reagents used were human-recombined visfatin (Peprotech, USA), anti-CD36, SR-BI, SR-A, ABCG1 (Santa Cruz, USA), anti-visfatin, ABCA1 (Abcam, USA), secondary antibody (Cell Signaling, USA), visfatin ELISA Kit (Raybiotech, USA), Oil red O (ORO; Sigma, USA), fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin (Gibco, USA), ox-LDL (Yiyuan Biotech, China), Amplex Red Cholesterol Assay Kit, BCA Protein Assay Kit, total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) Reagent Kits (Invitrogen, USA), SYBR Green qPCR Supermix (Takara, Japan), small interfering RNA (siRNA) against mouse visfatin, and Transfection Reagent (Ambion, USA).

### Animal experiments

**Animals** Six-week-old male apolipoprotein E (ApoE) knockout (KO) mice and C57BL/6 J mice from Vital River, which were introduced from Jackson Laboratory, were housed at  $22\pm 2$  °C,  $55\pm 5$  % relative humidity, with a 12-h light/dark cycle. ApoE KO mice were fed with a western diet (21 % fat and 0.15 % cholesterol) for 16 weeks to establish atherosclerosis model, while C57BL/6 J mice on a common diet as a control group. All experimental mice were allowed access to food and water *ad libitum* and weighed every 4 weeks.

**Serum lipid measurement** The mice were anesthetized by intraperitoneal injection with 10 % chloral hydrate (350 mg/kg), following eyeball extirpation to collect their blood after a 16-week experiment. Serum was prepared by a conventional method and subjected to measure the concentrations of TC, TG, HDL-C, and LDL-C by enzymatic procedures.

**The protein levels of visfatin in mice** To determine the circulating levels of visfatin, mice plasma was prepared and then analyzed using an enzyme-linked immunosorbent assay (ELISA) method according to the operating instructions of visfatin ELISA kit. Meanwhile, the proteins of mice aorta were isolated and measured using Western blotting methods.

**Assessment of atherosclerotic lesion area** After feeding on a fat-rich diet for 16 weeks, all mice were sacrificed, and hearts were perfused with iced phosphate-buffered saline (PBS) for 5 min. The aortic sinus was embedded and then

made into serial cryosections of 7- $\mu$ m thickness. The atherosclerotic lesions at aortic sinus were stained with ORO as previously described (Van Eck et al. 2000). The mean lesion area for each mouse was calculated from four sections. Whole aortic atherosclerotic lesions were determined by en face preparation followed by ORO staining as previously described (Kamari et al. 2011).

All animal experiments were carried out in accordance with the international guidelines and approved by the Ethics Committee of Southern Medical University.

#### Cell culture

To check foamy cell formation in vivo, peritoneal macrophages were isolated from ApoE KO mice after a 4-week high-fat diet as previously described (Takahashi et al. 2003). Briefly, the mice were injected intraperitoneally with 3 % thioglycollate, and peritoneal exudates were collected after 4 days. Macrophages were cultured in DMEM containing 10 % FBS. The nonadherent cells were removed after incubation for 3 h at 37 °C. The adherent cells were collected for ORO staining and cholesterol measurement. Murine macrophage RAW264.7 cells were cultured in DMEM supplemented with 10 % FBS, 1 % penicillin, and streptomycin.

#### Transfection of small interfering RNA

The sequences of siRNA against mouse visfatin were as follows: siRNA forward 5'-GGCACCACUAAUC AUCAGAtt-3' and reverse 5'-UCUGAUGAUUAG UGGUGCCtc-3'. Transfections of siRNA were performed as described for manual of transfection reagent. RAW264.7 cells were incubated for 24 h with siRNA and transfection reagent. Efficiency of visfatin silencing was analyzed at protein levels 2 days after transfection of siRNA.

#### ORO staining

Cells were washed by PBS and then fixed with 4 % paraformaldehyde for 15 min. After the removal of paraformaldehyde, cells were pretreated with 60 % isopropanol for several seconds and then stained with 0.3 % ORO for 10 min to visualize cellular lipid accumulation. Hematoxylin was used as counterstaining. Staining was recorded on a Nikon microscope equipped with a digital camera. The density of lipid content was evaluated by alcohol extraction after ORO staining. The absorbance at 540 nm was determined with a microplate reader.

#### Cholesterol measurement

RAW264.7 cells were treated with various concentrations of visfatin for 24 h and then washed twice with PBS. The

cellular TC was extracted by hexane/isopropanol. After centrifuging at 12,000 $\times$ g, the supernatants were captured and then dried under nitrogen flush and redissolved in isopropanol. The content of TC was determined with cholesterol assay kit. The cellular total protein was measured by BCA assay kit.

#### Western blotting

The mice aorta or cultured cells were lysed in a lysis buffer containing 50 mM Tris (pH7.4), 150 mM NaCl, 1 % NP-40, and 1 mM PMSF for 30 min on ice. After centrifugation at 12,000 $\times$ g for 15 min at 4 °C, the supernatants were collected as total proteins in aorta or cells. The protein concentrations were also determined with a BCA assay kit. Aliquots (50  $\mu$ g) of protein samples were separated on 10 % SDS-PAGE and electro-transferred to polyvinylidene fluoride membranes (PVDF, Millipore, Bedford, MA). The PVDF membranes were incubated with primary antibodies (Abs) overnight at 4 °C after being blocked with 5 % nonfat milk and then subjected to secondary Abs for 1~2 h at 37 °C. The protein bands were detected with an enhanced chemiluminescence system (ECL, CST) on Kodak 2000MM. Densitometric analysis was conducted by Molecular Imaging Software Version 4.0. Actin proteins were detected as a control.

#### Quantitative real-time PCR

Total cellular RNA was isolated from RAW264.7 cells using TRIzol reagent and converted into cDNAs by Thermoscript reverse transcriptase PCR system. cDNAs were quantified using SYBR Green qPCR Supermix supplemented with specific primers for mouse SR-A (5'-TGGTCCACCT GGTGCTCC-3' forward and 5'-ACCTCCAGGGA AGCCAAT TT-3' reverse), CD36 (5'-CAGTTGGAG ACCTGCTTATCC-3' forward and 5'-GCGTCCTGGGT TACATTTTC-3' reverse) and mouse  $\beta$ -actin (5'-TTGTC CCTGTATGCCTCTGG-3' forward and 5'-GAGGT CTTTACGGATGTCAACG-3' reverse) on MX3005P quantitative real-time PCR (qRT-PCR) system. Expression of target genes was measured in triplicate and then normalized to the level of the housekeeping gene  $\beta$ -actin. All the data were analyzed by MXPro 4.01 software.

#### Statistical analysis

All values are reported as mean  $\pm$  SEM values and analyzed with SPSS 13.0 version. Student t test was used to compare two independent groups, while one-way analysis of variance followed by student Newman-Keuls *q* post hoc analyses for multiple groups. Differences were considered significant at  $P < 0.05$ .

## Results

### Expression of visfatin in plasma and atherosclerotic lesions in ApoE KO mice

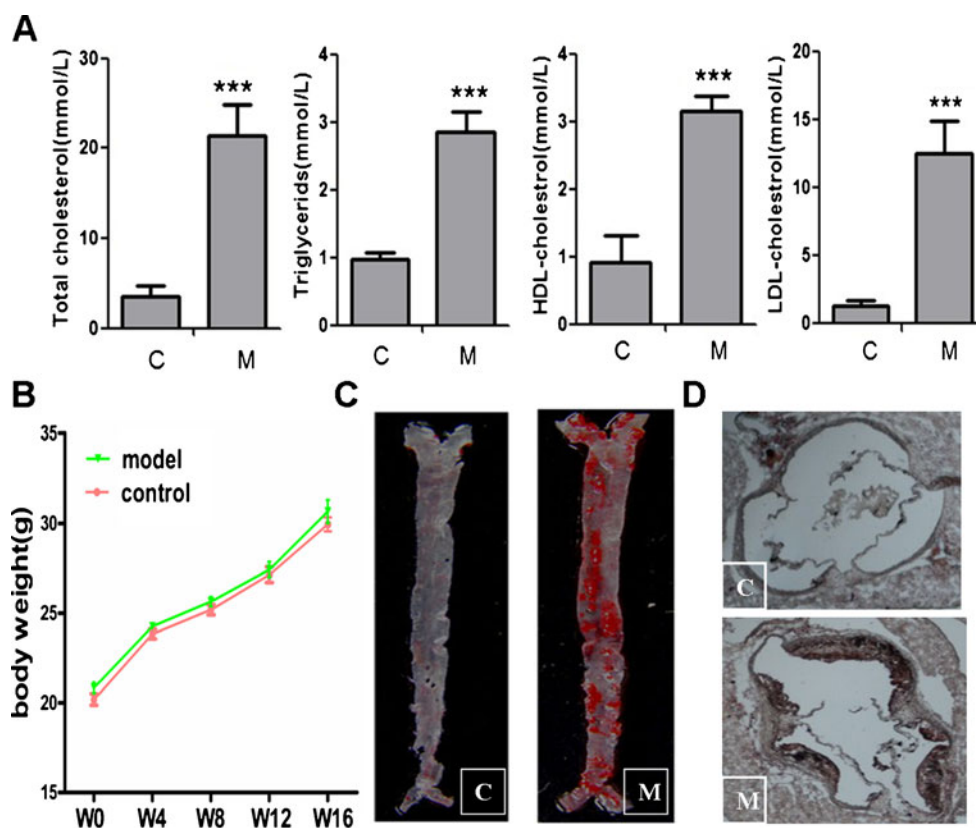
As we know, ApoE KO mouse is a well-established animal model to study atherosclerosis. The mice placed on a western diet gradually develop all the phases of atherosclerotic lesions from fatty streak to atheromatous plaque, which simulates the atherogenesis of humans under the nature state (Plump et al. 1992; Nakashima et al. 1994). After fed with high-fat diet for 16 weeks, the levels of serum lipids in ApoE KO mice, such as TC, TG, HDL-C, and LDL-C, were much higher than that in C57BL/6 J mice (Fig. 1a), while the body weight was not significantly different in these two groups (Fig. 1b). To evaluate the atherosclerotic lesion, whole aorta and aortic sinus were stained with ORO simultaneously and then quantitative analyzed by Image-Pro plus 6.0. The atherosclerotic plaques were much

noticeable in both whole aorta and aortic sinus in ApoE KO mice, while there was no obvious lesion in control group (Fig. 1c, d).

To analyze visfatin expression in mouse atherosclerosis model, we determined its level in both plasma and atherosclerotic lesions, respectively, by ELISA and Western blotting assay. In Fig. 2, we found that both plasma visfatin level and visfatin protein in aorta in the model group were much higher than that in control group. The protein level of visfatin in ApoE KO mice was almost 2.31 times that in C57BL/6 J mice (Fig. 2c). These results indicate that visfatin may play a role in the pathogenesis of atherosclerosis, which is also consistent with the previous research (Dahl et al. 2007).

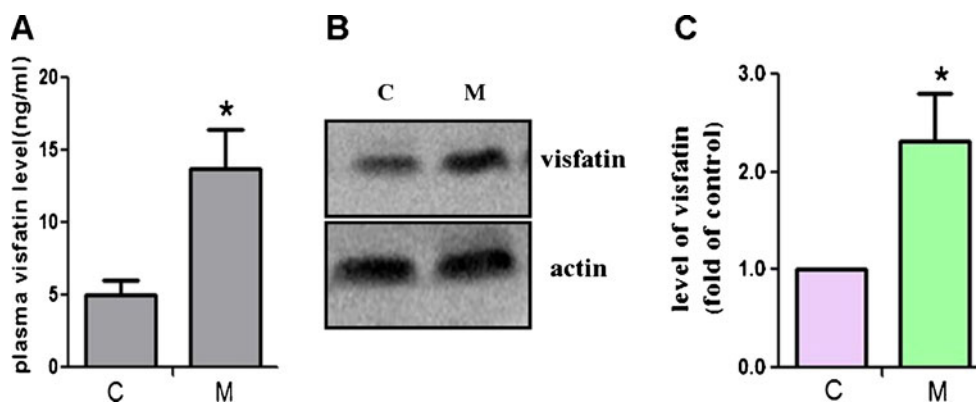
### Effect of ox-LDL on visfatin expression in RAW264.7 cells

It is well known that modified LDL is pro-atherogenic in the development of atherosclerosis, particularly the



**Fig. 1** The serum concentrations of cholesterol and atherosclerotic plaques in ApoE KO mice. **a** Serum cholesterol including total cholesterol, triglycerides, HDL-C, and LDL-C were determined in both ApoE KO mice with a western diet (M, model) and C57BL/6 J mice with a common diet (C, control) for 16 weeks ( $n=6$ ). \*\*\* $P<0.0001$  vs control group. **b** Body weight of mice was measured at the beginning on a western diet (W0) and every 4 weeks (W4, W8, W12) till the whole experiment period (W16). The results are the average of eight

measurements.  $P>0.05$  vs control group. **c** Atherosclerotic plaques in whole aorta both in ApoE KO mice and C57BL/6 J mice after a 16-week experiment. Whole aorta was stained with 0.5 % ORO for 5 min after washing with iced saline. Then the aorta was exposed to 60 % isopropanol for another 5 min and photographed by a digital camera after staining. **d** Atherosclerotic lesions in mice aorta sinus after a 16-week experiment. Cryosections of aortic sinus was stained with ORO. Representative photographs are shown. Magnification $\times 200$



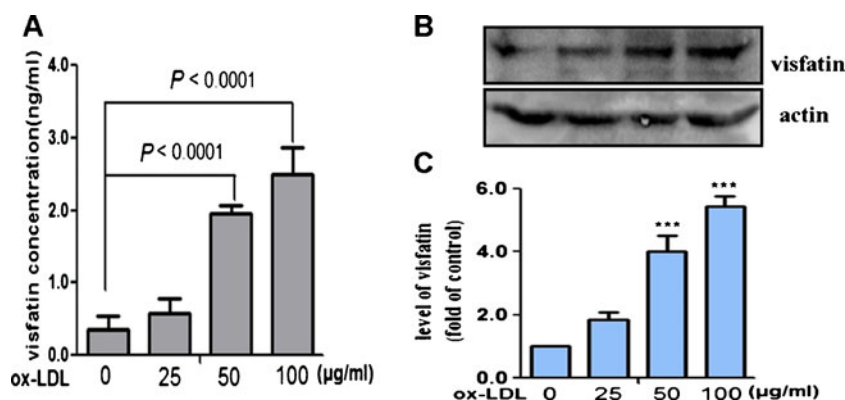
**Fig. 2** Visfatin expression is markedly increased in ApoE KO mice. **a** Plasma level of visfatin in ApoE KO mice with a western diet (M, model) and C57BL/6 J mice with a common diet (C, control) for 16 weeks was determined by ELISA assay ( $n=6$ ).  $*P<0.05$  vs control group. **b** The

protein expression of visfatin in mice aorta was determined by Western blot analysis. **c** Protein bands of visfatin and actin as a control were quantified by Molecular Imaging Software ( $n=3$ ).  $*P<0.05$  vs control group

important role of ox-LDL in cytokine secretion (Stemme et al. 1995). In this study, we examined for the first time whether ox-LDL could regulate visfatin expression in RAW264.7 cells. Mouse RAW264.7 cells are a macrophage-like cell line and also can be induced to form foam cells by ox-LDL (Li et al. 2009). Moreover, this cell line is more feasible for mechanistic studies compared with primary macrophages. We, thus, used RAW264.7 cells to form foam cells for further studies here. After pretreatment with ox-LDL (25, 50, or 100  $\mu\text{g/ml}$ ) for 24 h, the cell supernatant and protein were subjected to ELISA and Western blotting, respectively. In Fig. 3a, we saw that ox-LDL could dose-dependently increase visfatin release from cellular into supernatant culture. Furthermore, Western blotting of the protein samples also revealed a significant increase in visfatin expression by ox-LDL in a dose-dependent manner (Fig. 3b, c).

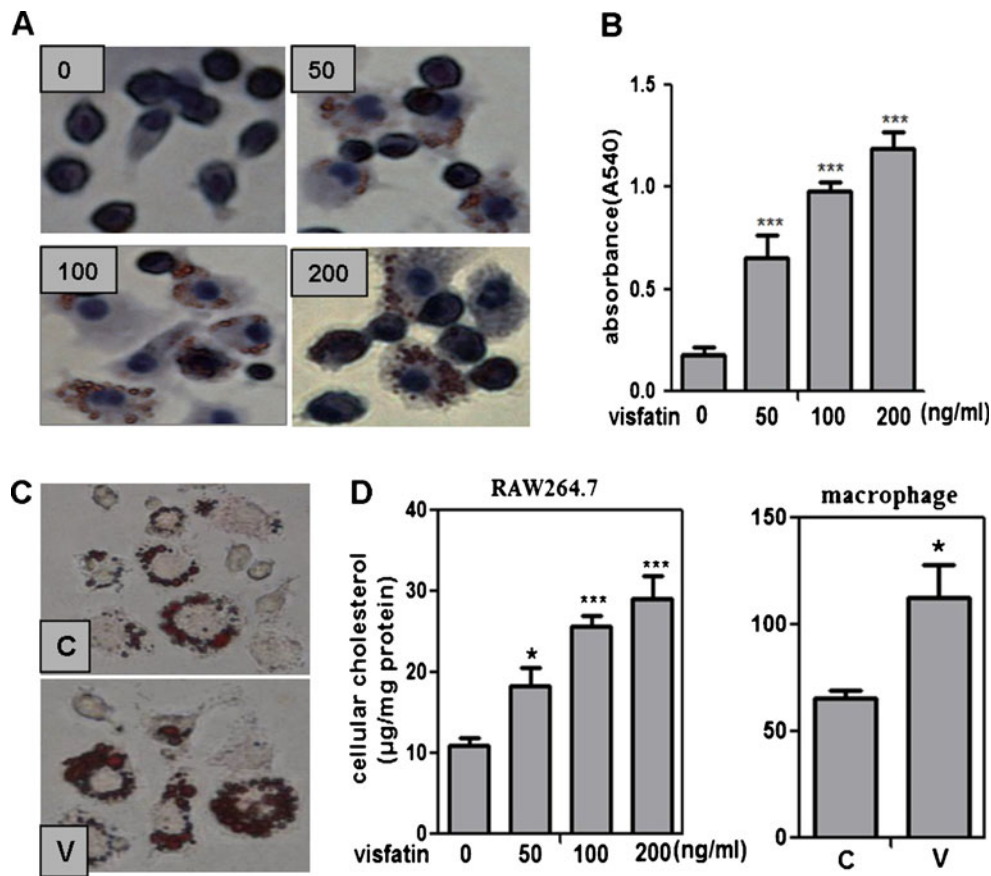
Visfatin induces cholesterol accumulation in macrophages both in vivo and in vitro

Though the study has indicated that visfatin may play a role in plaque destabilization, whether visfatin involved in the early foamy cell formation is largely unknown. As we have showed above, visfatin expression was markedly enhanced both in ApoE KO mice and ox-LDL-treated RAW264.7 cells. The molecular mechanism of visfatin in the formation of foam cells remains elusive. In our study, we found that visfatin could induce cholesterol accumulation in RAW264.7 cells (Fig. 4a, b). To evaluate the effect of visfatin in vivo foamy cells, six ApoE KO mice were treated with visfatin ( $15 \mu\text{g day}^{-1} \text{ mouse}^{-1} \text{ sc}$  for 8 days) after feeding with high-fat diet for 4 weeks. Notably, in accordance with excessive cholesterol accumulation induced by visfatin in RAW264.7 cells, we found that pretreatment with visfatin showed more lipids loaded in peritoneal macrophages than that in mice without



**Fig. 3** ox-LDL could dose-dependently increase visfatin expression in RAW264.7 cell line. RAW264.7 cells were exposed to different concentrations of ox-LDL (25, 50, or 100  $\mu\text{g/ml}$ ) for 24 h. **a** The level of visfatin secretion into the culture medium was measured by ELISA

assay ( $n=3$ ),  $P<0.0001$  vs untreated group. **b** and **c** Western blot analysis of visfatin expression in RAW264.7 cells incubated with ox-LDL.  $n=3$ ,  $***P<0.0001$  vs untreated group



**Fig. 4** Visfatin induces lipid accumulation in macrophages both in vivo and in vitro. **a** and **b** RAW264.7 cells were incubated with different doses of visfatin (50, 100, or 200 ng/ml) for 24 h. After fixation by 4 % paraformaldehyde, cells were stained with 0.5 % ORO to detect lipid accumulation. Cellular nuclei were stained with hematoxylin.  $n=6$ ,  $***P<0.0001$  vs untreated group. Magnification  $\times 400$ . **c** Visfatin accelerates lipid accumulation in peritoneal macrophages in visfatin-treated mice. After feeding with high-fat diet for

4 weeks, ApoE KO mice were treated with visfatin at the concentration of  $15 \mu\text{g day}^{-1}$  (*V*) or not (*C*) for another 8 days, and then peritoneal macrophages were collected to detect lipid accumulation by staining with ORO ( $n=6$ ). **d** Cellular cholesterol content was determined by an enzymatic method in RAW264.7 cells and peritoneal macrophages. The results are the average of six mice.  $*P<0.05$ ,  $***P<0.0001$  vs untreated group

visfatin treatment (Fig. 4c). Moreover, the content of TC in visfatin-treated mice was higher than that in the control mice, which is consistent with the result in RAW264.7 cells (Fig. 4d). All of these results suggest that visfatin promotes the formation of foam cells both in vivo and in vitro, which is mainly due to increasing cholesterol uptake in macrophages.

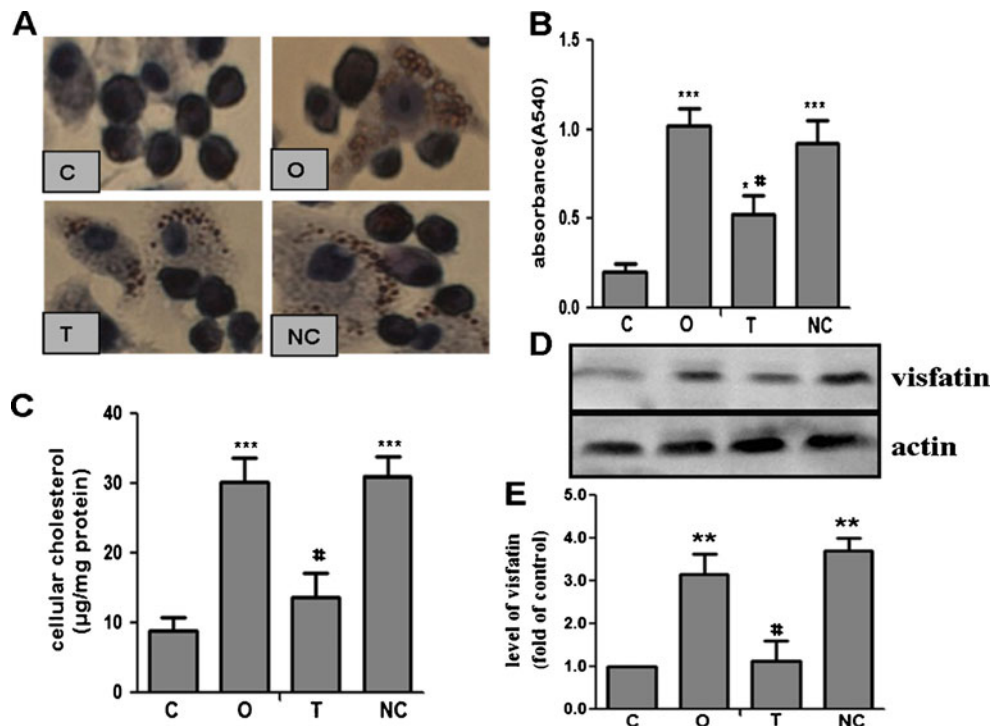
Cholesterol uptake was reduced in RAW264.7 cells after visfatin downregulation by RNAi

To study whether cholesterol accumulation in the treatment of ox-LDL is visfatin-dependent, we downregulated visfatin expression in RAW264.7 cells using RNA interference. After pretreatment with ox-LDL (50  $\mu\text{g/ml}$ ) for 24 h, we found that RAW264.7 cells with downregulated visfatin expression exhibited significantly lower levels of cholesterol uptake (Fig. 5a–c). The efficiency of silencing of visfatin gene expression by RNAi was evaluated at the protein level

by Western blot analysis. Levels of visfatin protein was significantly downregulated after RNAi treatment compared to cells transfected with a nonsense sequence as negative control (Fig. 5d, e). These results indicate that cholesterol uptake of RAW264.7 cells in ox-LDL treatment was largely regulated by the effects of visfatin.

Visfatin induces lipid uptake by activation of SR-A and CD36

To further clarify the underlying mechanism of visfatin in promoting foamy cell formation, the macrophages SR and RCT were evaluated after treatment with visfatin. As we all know, SR-A, CD36, SR-BI, ABCA1, and ABCG1 have been established to play crucial keys in cholesterol homeostasis during formation of foam cells (Kunjathoor et al. 2002; Van et al. 2004; Wang et al. 2001; Out et al. 2006). As shown in Fig. 5a, visfatin could induce the activation of SR-A and CD36, which are responsible for the lipid uptake



**Fig. 5** Cholesterol uptake was reduced in RAW264.7 cells after visfatin downregulation by RNAi. Cells were divided into four groups as follows: control group (C), ox-LDL group (O), transfection of siRNA group (T), and negative control group (NC). Cells in the latter three groups were pretreated with ox-LDL (50 µg/ml) for 24 h, then were subjected to transfection or not. **a** and **b** Lipid accumulation in RAW264.7 cells were analyzed by ORO staining after transfection of siRNA. Hematoxylin was used as counterstaining.  $n=6$ ,  $*P<0.05$ ,

$***P<0.0001$  vs control group,  $\#P<0.05$  vs ox-LDL group. Magnification  $\times 400$ . **c** Cellular cholesterol content was determined after visfatin downregulation in RAW264.7 cells.  $***P<0.0001$  vs control group,  $\#P<0.05$  vs ox-LDL group. **d** and **e** The efficiency of visfatin silencing was analyzed 48 h after transfection of siRNA by Western blotting. The actin was used as the quantitative standard.  $n=3$ ,  $**P<0.01$  vs control group,  $\#P<0.05$  vs ox-LDL group

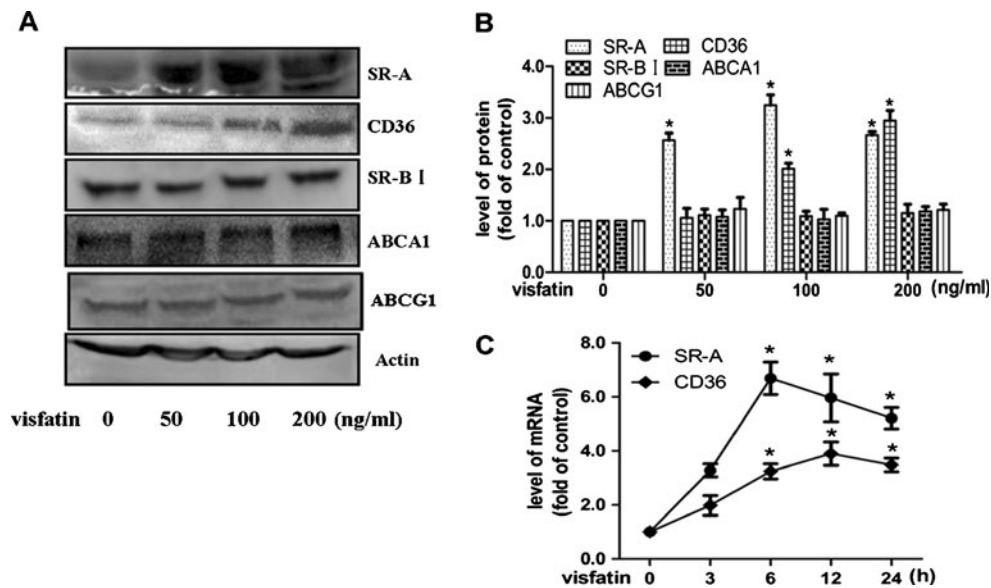
by macrophages. On the other hand, visfatin showed no effect in expression of SR-BI, ABCA1, or ABCG1, which mainly maintain the course of reverse cholesterol transport (Fig. 6a, b). Furthermore, to better understand the effect of visfatin on the expression of SR-A and CD36, RAW264.7 cells were exposed to visfatin (100 ng/ml) for 3, 6, 12, and 24 h. In accordance with the promotion in protein level, we found that visfatin also markedly increased mRNA levels of SR-A and CD36, reaching a maximum at 6 and 12 h, respectively (Fig. 6c). These data indicate that visfatin accelerates foamy cell formation through modulating expression of SR-A and CD36.

## Discussion

Visfatin (also known as PBEF) was first reported to be secreted by activated lymphocytes in bone marrow stromal cells (Samal et al. 1994). Over the past several years, a series of evidence has indicated that it is also ubiquitously present in most other tissues, such as adipose tissue, skeletal muscle, spleen, and liver (Sethi and Vidal-Puig 2005). Visfatin could stimulate the release of cytokines and is induced by

inflammatory stimuli such as TNF- $\alpha$ , LPS, and IL-6 (Jia et al. 2004; Nowell et al. 2006). As a multifunctional adipokine, visfatin has also been implicated in the pathogenesis of obesity, diabetes mellitus, hyperlipidemia, and such atherosclerosis-related diseases. Due to the close association between visfatin and inflammation, Dahl and his colleagues studied the effect of visfatin in macrophages of humans with unstable carotid and coronary atherosclerosis. They have identified that visfatin is a potential inflammatory mediator in plaque destabilization. Another study has further demonstrated that the regulation of visfatin in macrophages is related to pro-atherogenic stimuli, including hypoxia, TNF- $\alpha$  and ox-LDL (Dahl et al. 2011). The pro-inflammatory effect of visfatin in macrophages has been well established. However, the effect of visfatin and its possible mechanism involved in cholesterol metabolism of macrophage-derived foam cells remained elusive.

In the present study, we revealed the novel molecular mechanisms underlying the pro-atherogenic action of visfatin in foamy cell formation during development of atherosclerosis. Firstly, we found that plasma visfatin concentrations were significantly increased in atherosclerotic mice with the elevation of serum lipid level (Figs. 1 and 2).



**Fig. 6** Effects of visfatin on the expression of SR-A, CD36, SR-BI, ABCA1, and ABCG1. **a** RAW264.7 cells were incubated with visfatin (50, 100, or 200 ng/ml) for 24 h. Cell samples were then subjected to Western blotting to detect the protein levels of SR-A, CD36, SR-BI, ABCA1, ABCG1, and actin. **b** Protein bands of SR-A, CD36, SR-BI, ABCA1, ABCG1, and actin as a control were analyzed by quantified

Software. Data are mean  $\pm$  SEM from three independent experiments. \* $P < 0.05$  vs untreated group. **c** Effects of visfatin on mRNA level of SR-A and CD36. RAW264.7 cells were exposed to visfatin (100 ng/ml) at different time points (0, 3, 6, 12, or 24 h). Total cellular RNA was collected and then subjected to qRT-PCR to determine mRNA level of SR-A and CD36 ( $n = 3$ ). \* $P < 0.05$  vs untreated group

This finding indicates that circulating levels of visfatin are increased during hyperlipidemia state. Western blotting of protein samples in mouse aorta also showed a similar increase in visfatin expression, which is consistent with previous studies in patients with carotid atherosclerosis. In this study, to our best knowledge, we demonstrate for the first time that both circulating and protein levels of visfatin are markedly promoted in atherosclerotic mice. Our finding further implies that visfatin may play an important role in the pathogenesis of atherosclerosis.

Accumulation of modified LDL such as ox-LDL in macrophages is a key event in all stages of atherogenesis. Previous studies have reported that cholesterol accumulation in macrophages could be regulated by mediating expression of SRs and RCTs or inflammatory responses by cytokine induction (Glass and Witztum 2001; Berliner and Heinecke 1996). Here, we found that ox-LDL pretreatment increased visfatin expression both in a supernatant culture and within RAW264.7 cells in a dose-dependent manner (Fig. 3). This finding is consistent with the previous report in THP-1 cells (Dahl et al. 2007, 2011). On the basis of our findings in vitro, such a pro-atherogenic factor could be a potent stimulus for visfatin expression, potentially explaining its enhanced expression in plasma and atherosclerotic plaques. In addition, to clarify the effect of visfatin on lipid metabolism in macrophages, we determined the level of cellular cholesterol induced by visfatin both in cultured cells and in mice (Fig. 4). Lipid accumulation in RAW264.7 cells was induced by visfatin in a dose-dependent manner. Furthermore, RNA

interference against visfatin significantly reduced the lipid uptake of RAW264.7 cells (Fig. 5). Peritoneal macrophages from ApoE KO mice treated with visfatin for 8 days were more lipid-loaded than did that from the control mice. Although some of our findings were rather modest, our data may suggest that exogenous visfatin promotes accumulation of unwanted self-lipids in macrophages and accelerates foamy cell formation induced by ox-LDL and other pro-atherogenic stimuli in vivo, further supporting a link between visfatin and atherogenesis.

SR-modulated ox-LDL internalization and RCT-dependent cholesterol efflux have been demonstrated to be crucial events in the maintenance of intracellular cholesterol homeostasis of macrophages (Kleemann et al. 2008; Cheng et al. 2011; Kunjathoor et al. 2002; Ji et al. 2011). Therefore, we examined the regulatory effect of visfatin on SRs and RCTs. Our data show that visfatin induced a marked increase in SR-A and CD36, but not SR-BI, ABCA1, and ABCG1 (Fig. 6). Here, we may be the first to report that acceleration of cholesterol accumulation by visfatin during formation of foam cell is mainly associated with ox-LDL uptake. Both SR-A and CD36 play a key role in the uptake of ox-LDL during the development of atherosclerosis (Kunjathoor et al. 2002). It was reported recently that antiatherogenic antioxidants could ameliorate the formation of foam cells through transcriptional regulation of SR-A (Tsai et al. 2010). In the current study, our observations suggest that both transcriptional and posttranscriptional regulation may work in concert to mediate the expression of SR-A and CD36 in visfatin-treated cells,



indicating that the pro-atherogenic action of visfatin is mainly due to regulation of the expression of these two receptors.

Accumulating evidences have shown that some signaling pathway may be implicated in the uptake of ox-LDL by SR-A and CD36, including MAPK, PPAR, PI3K, and so on (Rahaman et al. 2006; Xie et al. 2011; Li et al. 2009). CD36 was found to be mediated by Lyn and MEKK2 signaling pathway that were activated by ox-LDL specifically in macrophages. In view of the function of these two receptors, the increase in their expression in visfatin-induced macrophages likely contributes to elevating ox-LDL uptake and subsequently promoting the development of foam cells. Although the current study has some limitations (e.g., some of the findings were rather modest, we did not test visfatin inhibitor in all experiments), our findings may suggest that visfatin promotes cholesterol accumulation in macrophages and atherogenesis through modulating expression of SR-A and CD36. The signaling pathway implicated in the activation of SR-A and CD36 induced by visfatin is under investigation.

In summary, we propose new insights into the pro-atherogenic effect of visfatin in promoting cholesterol accumulation during the development of foam cells by up-regulating the expression of SR-A and CD36 in macrophages. We are willing to provide some new information for better understanding on the potential effect of visfatin in accelerating foamy cell formation in atherosclerosis. However, whether visfatin will eventually become a therapeutic target warrants further investigation.

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**Conflict of interests** The authors declare that there is no conflict of interests.

## Reference

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