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Visfatin enhances ICAM-1 and VCAM-1 expression through ROS-dependent NF-κB activation in endothelial cells

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Abstract

Visfatin has recently been identified as a novel visceral adipokine which may be involved in obesity-related vascular disorders. However, it is not known whether visfatin directly contributes to endothelial dysfunction. Here, we investigated the effect of visfatin on vascular inflammation, a key step in a variety of vascular diseases. Visfatin induced leukocyte adhesion to endothelial cells and the aortic endothelium by induction of the cell adhesion molecules, ICAM-1 and VCAM-1. Promoter analysis revealed that visfatin-mediated induction of CAMs is mainly regulated by nuclear factor-κB (NF-κB). Visfatin stimulated IκBα phosphorylation, nuclear translocation of the p65 subunit of NF-κB, and NF-κB DNA binding activity in HMECs. Furthermore, visfatin increased ROS generation, and visfatin-induced CAMs expression and NF-κB activation were abrogated in the presence of the direct scavenger of ROS. Taken together, our results demonstrate that visfatin is a vascular inflammatory molecule that increases expression of the inflammatory CAMs, ICAM-1 and VCAM-1, through ROS-dependent NF-κB activation in endothelial cells.

Keywords: Cell adhesion molecules; NF-κB; Reactive oxygen species; Vascular inflammation; Visfatin

1. Introduction

The inflammatory reaction involves complex interactions between inflammatory cells (neutrophils, lymphocytes, and monocytes/macrophages) and vascular cells (endothelial cells and smooth muscle cells). Vascular cells regulate the inflammatory process through the expression of adhesion molecules, cytokines, chemokines, and growth factors [1–3]. Endothelial cell adhesion molecules (ECAMs), such as E-selectin, VCAM-1, and ICAM-1, are up-regulated in the vascular endothelium in response to inflammatory stimuli, which in turn mediates leukocyte recruitment and adherence to the endothelium at the early stages of vascular inflammation, ultimately leading to the progression of numerous vascular diseases [4].

Adipose tissue is a dynamic paracrine and endocrine organ that secretes several hormones and cytokines (termed "adipokines"). These adipokines function in the pathogenesis of the insulin resistance syndrome, and directly affect vascular function [5]. Adipokines such as resistin and leptin are proinflammatory

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mediators that directly contribute to endothelial dysfunction and atherogenesis [6,7].

Visfatin (also known as pre-B-cell colony-enhancing factor, PBEF) is a novel adipokine that is preferentially produced by visceral adipose tissue and has insulin-mimetic actions [8]. It also functions as a proinflammatory adipocytokine that is secreted by neutrophils in response to inflammatory stimuli and upregulates the production of cytokines in the monocytes [9,10], suggesting a potential role in the pathogenesis of inflammatory disorders. We have previously reported that visfatin promotes angiogenesis [11] which can be associated with obesity-related vascular diseases, including atherosclerosis, diabetes, and hypertension [12,13].

In this study, we hypothesized that visfatin directly promotes endothelial dysfunction. Visfatin markedly increased the expression of ICAM-1 and VCAM-1, which bind leukocytes to endothelial cells through the activation of NF-κB, via the ROS production in endothelial cells.

2. Materials and methods

2.1. Reagents and recombinant proteins

N-acetyl-L-cysteine (NAC), oxypurinol, rotenone, diphenylene iodonium (DPI), pyrrolidine dithiocarbamate (PDTC), polymyxin B, and dihydroethidium (DHE) were obtained from Sigma. Calcein-AM was obtained from Molecular Probes. Mouse monoclonal anti-ICAM-1, mouse monoclonal anti-VCAM-1, rabbit polyclonal anti-NF-κB p65, mouse monoclonal anti-IκBα, and rabbit monoclonal anti-phospho-IκBα antibodies were obtained from Santa Cruz Biotechnology. Human α-tubulin antibody was purchased from Biogenex. FITC-conjugated donkey anti-rabbit IgG was purchased from Jackson Immunoresearch. The full-length human visfatin expression vector was constructed by PCR and subcloned into the pET vector (Novagen). For production of His fusion protein, pET-visfatin was transformed and His-visfatin protein was purified on the Talon metal affinity column (CLONTECH). This protein was dialyzed using the Sephadex G-25 column (Amersham Pharmacia Biotech). The eluted protein was desalted using a PD-10 Desalting column (Amersham Pharmacia Biotech) into phosphate-buffered saline (PBS). The endotoxin level in the recombinant visfatin was below 0.05 EU (<5 pg/ml) as determined by *Limulus* amebocyte lysate testing (Associates of Cape Cod, Inc.). The amount of LPS present in our visfatin preparation was below 5 pg/ml, whereas the critical amount to induce ICAM-1 and VCAM-1 gene expression in endothelial cells exceeded 100 pg/ml [14,15].

2.2. Cell culture

Human microvascular endothelial cells (HMECs) were obtained from the CDC (Atlanta, GA). These cells were maintained in MCDB supplemented with 10% FBS (Invitrogen), 1% antibiotics, 1 μg/ml hydrocortisone, and 10 ng/ml hEGF at 37 °C under a humidified 95% to 5% (vol/vol) mixture of air and CO_2. Primary human umbilical vein endothelial cells (HUVECs) (passage 5–8) were purchase from CLONECTICS. The HUVECs were plated onto 0.3% gelatin-coated dish and grown in M199 (Invitrogen) with heat-inactivated 20% FBS (Invitrogen), 3 ng/ml bFGF, and 100 μg/ml heparin. U937 monocytic cells were grown in RPMI-1640 (Invitrogen) with 10% FBS (Invitrogen) and 1% antibiotics.

2.3. In vitro monocyte adhesion assay

HMECs were plated on 24 well plates at 5×10^4 cells/well and incubated with or without visfatin for 8 h. U937 cells were then added (5×10^5 cells/ml, 200 μl/well) to the confluent monolayers of HMECs and incubated for 30 min. Non-adherent monocytes were removed by washing twice with PBS. Adherent cells were fixed and washed twice with PBS. Binding of monocytes to the endothelial cells were stained with Diff-Quick (Sysmex), and the adherent cells were counted in 3 separate fields in each well by microscopy (Nikon). The average number of adherent monocytes was calculated in the three fields for each set of four wells.

2.4. Ex vivo monocyte adhesion assay

The *ex vivo* binding of monocytes to the aorta dissected from rats was examined by *ex vivo* monocyte binding assay [16]. Male Sprague–Dawley rats (SD, 6 weeks of age), weighing 210–230 g, were obtained from Samtako, Osan, Korea. All animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23 revised 1996). The aortas were opened longitudinally, and incubated with visfatin for 16 h. The aortas were then incubated for 30 min with 1×10^6 fluorescence-labeled (using Calcein-AM) monocytes. After incubation, unbound monocytes were rinsed away; the adherent monocytes were counted in 3 consistent fields using fluorescence microscopy (Nikon).

2.5. In vivo assessment of macrophage adhesion and infiltration in vivo

Peritoneal macrophage was isolated with intraperitoneal stimulation of 4% triglycolate for four days. The macrophages were plated after washing them with RPMI twice. After 15 h, floating cells were removed and the attached macrophages were labeled with Cell tracker-Red (Molecular Probes) according to the manufacturer's instruction. The labeled cells (5×10^4) were administrated to a tail vein and fluorescence imaging was noninvasively performed through the mouse ear where 10 μl of visfatin (1 mg/ml) or saline was subcutaneously delivered 15 h before the imaging acquisition. Vasculature was episcopically visualized under a fluorescence intravital microscope.

2.6. RT-PCR

Total RNA was isolated from HMECs with a TRIzol reagent kit (Invitrogen). cDNA synthesis was performed on 3 μg of total RNA with a reverse transcription kit (Promega). The oligonucleotide primers for PCR were designed as follows: β-actin, 5′-GACTACCTCATGAAGATC-3′ and 5′-GATCCACATCTGCTGGAA-3′; ICAM-1, 5′-CGATGACCATCTACAGCTTTCCGG-3′ and 5′-GCTGCTACCACAGTGATGATGACAA-3′; VCAM-1, 5′-GATACAACCGTCTTGGTCAGCCC-3′ and 5′-CAGTTGAAGGATGCGGGAGTATATG-3′.

2.7. Real-time PCR

Real-time PCR quantification was performed using a SYBR Green approach (Light Cycler; Roche Applied Science, Penzberg, Germany). Cycling parameters consisted of 1 cycle of 95 °C for 10 min, followed by amplification for 30 cycles of 95 °C for 10 s, 57 °C for 5 s, and 72 °C for 7 s. Subsequently, a melting curve program was applied with continuous fluorescence measurement. The entire cycling process including data analysis took less than 1 h and was monitored using the LightCycler® software program (version 4.0).

2.8. Western immunoblot analysis

Harvested cells were lysed in a lysis buffer (40 mM Tris–Cl, 10 mM EDTA, 120 mM NaCl and 0.1% NP-40 with protease inhibitor cocktail (Sigma)). A constant protein concentration (30 μg/lane) was used. Proteins were separated by SDS/PAGE and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane was blocked with 5% skim milk in PBS containing 0.1% Tween-20 for 1 h at room temperature and probed with appropriate antibodies. This signal was developed with the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech).

2.9. Immunocytochemistry

Cells cultured on a coverglass were fixed in 4% paraformaldehyde for 10 min, blocked with 0.5% Triton X-100/PBS for 5 min, and then labeled with the appropriate primary antibodies and FITC-conjugated secondary antibody. Coverslips were mounted in Vectastain containing DAPI (Vector Laboratories). Cells were analyzed using fluorescence microscopy (Nikon).

2.10. Transient transfection and reporter gene analysis

Cells were plated on 24-well plates and transfected with the luciferase construct and pCMV-β-gal using Lipofectamine plus reagents (Invitrogen). Cell extracts were prepared 48 h after transfection, and then analyzed with the β-galactosidase enzyme assay for luciferase activity using an assay kit (Promega) and a luminometer (Tuner Biosystems). Each extract was assayed at least three times and relative luciferase activity was calculated as RLU/β-galactosidase. ICAM-1 and VCAM-1 luciferase reporter constructs with full-length (ICAM-1: −1350 to +45 bp, VCAM-1: −1716 to +119 bp) and truncated forms (ICAM-1: −485 to +45 bp, VCAM-1: −213 to +119 bp) were used as previously reported [17].

2.11. Nuclear extraction and EMSA

Nuclear extracts from HMECs were prepared and analyzed by EMSA as previously described [18]. Binding reactions containing equal amounts of protein (10 μg) and 20 fmol of biotin-labeled NF-κB oligonucleotide (probe) were performed for 20 min in binding buffer (10 mM Tris–Cl, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.05% NP-40, 1 μg poly (dI·dC) and 2.5% glycerol). Binding reactions were analyzed using 6% native PAGE. After blotting to a nylon membrane, labeled oligonucleotides were detected with the LightShift Chemiluminescent EMSA Kit following the instructions of the manufacturer (Pierce).

2.12. Determination of intracellular ROS

Intracellular ROS were detected in cultures using the oxidant-sensitive fluorogenic probe dihydroethidium (DHE), as previously described [19]. After loading with DHE (10 μM) for 30 min at 37 °C, cultures were treated with visfatin for 20 min. After these treatments, cultures were washed once with PBS, and ethidium-positive cells were detected using a fluorescence microscope (Nikon) equipped with a digital camera.

2.13. Measurement of NAD(P)H oxidase activity

NAD(P)H oxidase activity was measured with the lucigenin assay which is specific for superoxide. Cells were homogenized with a lysis buffer containing protease inhibitor (20 mM monobasic potassium phosphate, pH 7, 1 mM EGTA). NAD(P)H oxidase was determined in a 50 mM phosphate buffer, pH 7.0, containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin as the electron acceptor, and 100 μM NAD(P)H as the substrate [20]. The reaction was started

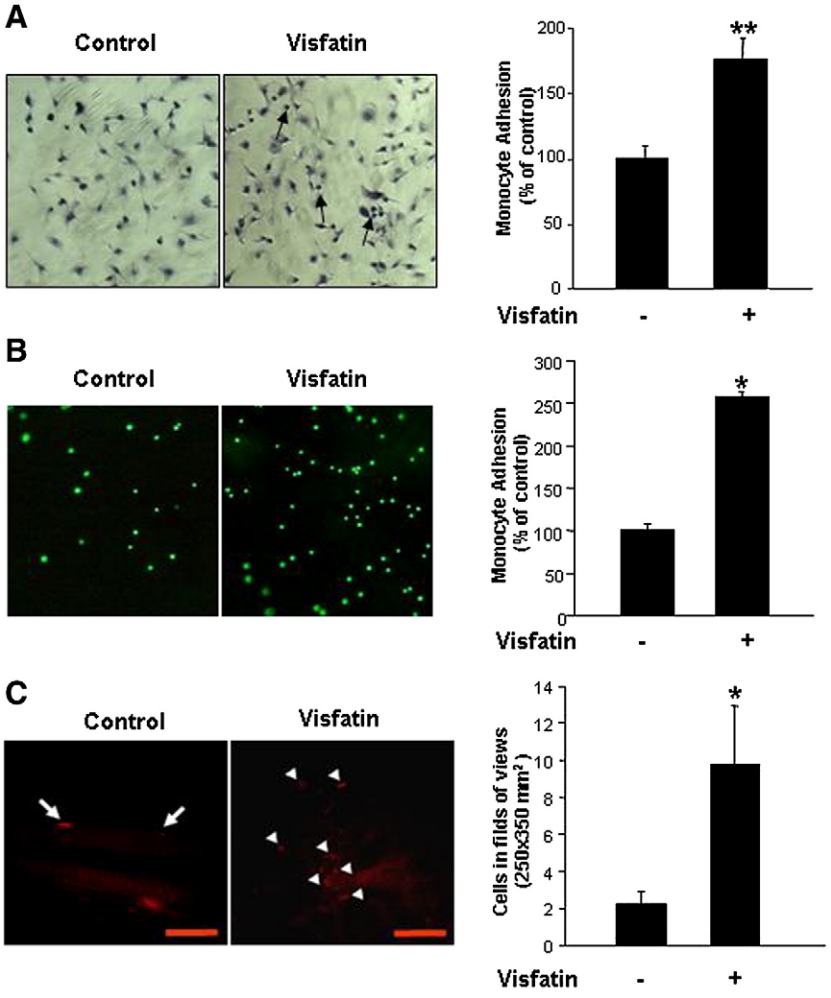


Fig. 1. Visfatin stimulates leukocytes adhesion to endothelial cells *in vitro* and *in vivo*. A, HMECs were incubated with or without visfatin (500 ng/ml) for 8 h, followed by measuring the adhesion of U937 monocytes as described in "Materials and methods". Arrows indicate the adhesion of U937 monocytes to HMECs. B, Adhesion of fluorescence-labeled monocytes to the endothelium of rat aorta. Calcein-AM stained monocytes were identified by fluorescence microscopy at 40× magnification. Data is the mean±SE relative to adhesion of untreated cells (set at 100%) in triplicate experiments. C, Imaging assessment of the macrophage adhesion and infiltration was performed at 2 h after administration of the labeled macrophage. The visfatin-treated area of the mouse ear showed increased adhesion and infiltration (arrowhead) compared with the vehicle-treated group where the basal level of adhesion (arrow) was observed (scale bar=50 μm). The macrophage staying in fields of views (250×350 μm^2) of nine images were counted and summarized. *$P<0.01$; **$P<0.05$ compared to control.

by addition of 25 μg protein, and proton emission was measured by using a luminometer (Turner Biosystems).

3. Results

3.1. Visfatin induces adhesion of leukocytes to endothelial cells in vitro and in vivo

We first investigated the effect of visfatin on the adhesion of leukocytes to endothelial cells, a critical step in vascular inflammation [4]. Treatment with visfatin (500 ng/ml) for 8 h enhanced the adhesiveness of U937 cells to HMECs (Fig. 1A) and HUVECs (Fig. S1). This dose of visfatin has been widely used to investigate the *in vitro* proinflammatory effects of visfatin in various cultured cells [9,21,22]. To determine whether visfatin induced monocyte binding to the *ex vivo* aorta, we performed an *ex vivo* adhesion assay using fluorescence-labeled monocytes and an aorta isolated from an SD rat. Fluorescent cells were observed on the surface of the aortic endothelium (Fig. 1B), and visfatin treatment increased the number of adherent cells by three times versus the control aorta. Next, to examine the effect of visfatin on macrophage adhesion *in vivo*, we introduced a noninvasive imaging system using an *in vivo* vital microscope as described in "Materials and methods". As shown in Fig. 1C, visfatin-treated mice showed a significant increase of macrophage adherence or infiltration to the blood vessels compared with vehicle-treated mice.

3.2. Visfatin increases ICAM-1 and VCAM-1 expression in endothelial cells

Cell adhesion molecules, such as ICAM-1 and VCAM-1, on endothelial cells mediate leukocyte adhesion to the endothelium during inflammation [1–3]. Therefore, we investigated whether visfatin could induce the expression of ICAM-1 and VCAM-1 on endothelial cells. Treatment of HMECs with visfatin increased the expression of ICAM-1 and VCAM-1 mRNA and the protein levels in a dose-dependent manner (Fig. 2A, left and B). Using real-time RT-PCR, we also quantified the expression levels of ICAM-1 and VCAM-1 mRNA enhanced by visfatin in the presence of 10 μg/ml endotoxin-neutralizing agent polymyxin B (Fig. 2A, right).

3.3. Visfatin induces the promoter activity of ICAM-1 and VCAM-1 genes through NF-κB

NF-κB is a potent proinflammatory nuclear transcription factor, and activation of NF-κB is a central event in the initiation and amplification of inflammatory responses [23]. Both ICAM-1 and VCAM-1 promoters contain NF-κB binding sites. The proximal NF-κB binding sites located ~200 bp (ICAM-1), and 65 and 75 bp (VCAM-1) upstream of the transcription start site are particularly important for the induction of ICAM-1 and VCAM-1 transcription [17,24]. To determine if NF-κB was involved in visfatin-mediated increases in ICAM-1 and VCAM-1, we transfected ICAM-1 and VCAM-1 luciferase reporter constructs, both

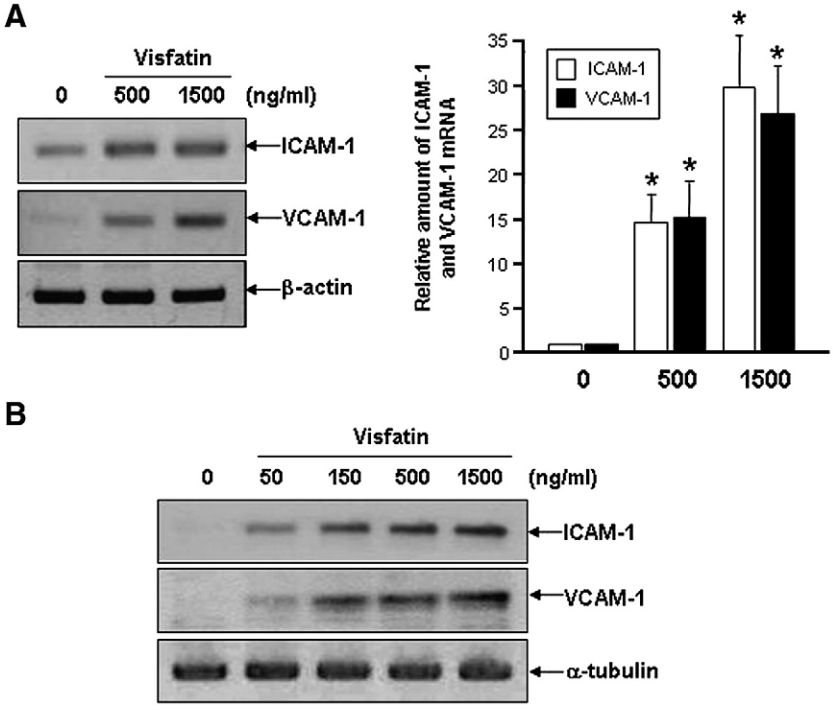


Fig. 2. Visfatin induces mRNA and protein expression of ICAM-1 and VCAM-1 in HMECs. HMECs were incubated with or without visfatin (500 ng/ml) for 4 h (A) or 24 h (B). A, Total RNAs were isolated and then analyzed by RT-PCR using specific primers to human ICAM-1 and VCAM-1. β-actin served as an internal control (left). Using real-time RT-PCR, we also quantified the expression levels of ICAM-1 and VCAM-1 mRNA. Culture medium was supplemented with 10 μg/ml polymyxin B. The expression level of control (untreated) was set to 1, and the values are normalized to β-actin mRNA levels (right). *$P<0.01$ compared to control. B, ICAM-1 and VCAM-1 protein levels were examined by Western blotting using anti-ICAM-1 and anti-VCAM-1 antibodies. α-tubulin served as the loading control.

full-length and truncated forms, into HMECs. The full-length promoter regions of both adhesion molecules have several transcription factor binding sites, including NF-κB, ARE, and TATA for the 1.4 kb ICAM-1 promoter, and NF-κB, TRE, and GATA for the 1.8 kb VCAM-1 promoter (Fig. 3). Both the ICAM-1 and VCAM-1 truncated forms contain proximal NF-κB binding sites (Fig. 3). Visfatin significantly increased promoter activity in the truncated ICAM-1 and VCAM-1 promoters, which shows similar increase in the reporter activities of full-length ICAM-1 and VCAM-1 genes (Fig. 3A and B), indicating that NF-κB binding sites are essential and sufficient for the visfatin-induced transcriptional activation of ICAM-1 and VCAM-1 genes. Furthermore, pyrrolidine dithiocarbamate (PDTC), a potent inhibitor of NF-κB [25], reduced visfatin-induced ICAM-1 and VCAM-1 mRNA levels (Fig. 3C). Thus, the NF-κB binding site is directly involved in the visfatin-mediated activation of the ICAM-1 and VCAM-1 promoter.

3.4. Visfatin stimulates NF-κB activation in endothelial cells

The main activated form of NF-κB is a heterodimer of the p65 subunit (also called relA) associated with either a p50 or p52 subunit. IκBα regulates transient NF-κB activation through phosphorylation, ubiquitination, and proteolytic degradation of IκBα [26]. Therefore, we examined the effect of visfatin on IκBα phosphorylation and degradation using Western blot analysis with antibodies against phosphospecific IκBα (Ser-32) and IκBα. Visfatin induced phosphorylation of Ser-32 in IκBα, with a maximal effect after 20 min of visfatin stimulation (Fig. 4A). Some degradation of IκBα was observed at 30 min in HMECs (Fig. 4B). Visfatin also significantly increased the nuclear translocation of the p65 subunit of NF-κB (Fig. 4C). Nuclear extracts were prepared and subjected to an EMSA with a specific probe for the NF-κB binding site. As expected, visfatin stimulated the binding of nuclear extracts to the NF-κB

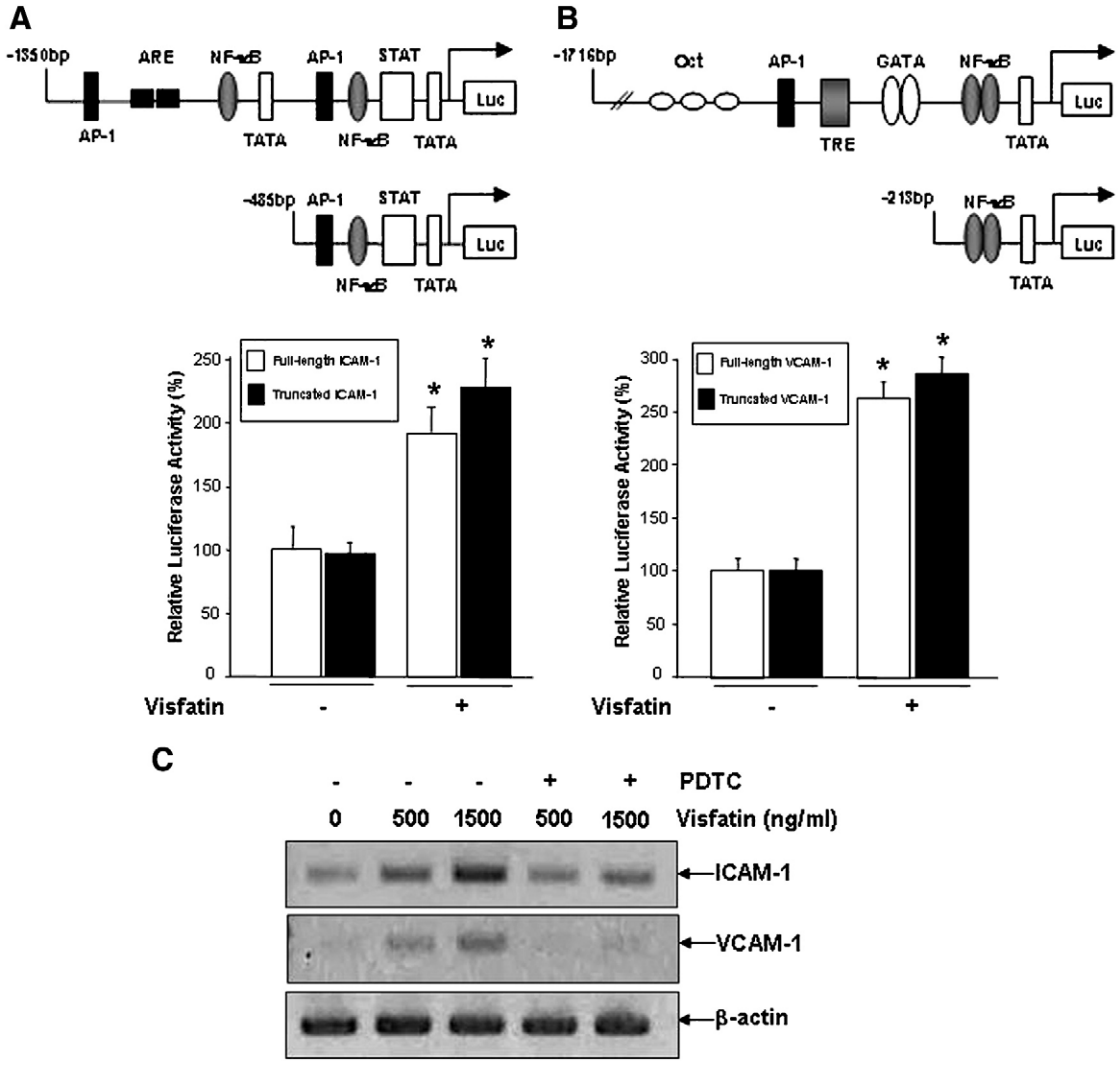


Fig. 3. Visfatin increases the promoter activities of ICAM-1 and VCAM-1 genes through NF-κB. A, B, Top, schematic representation of full-length and truncated (containing NF-κB motif) promoter of ICAM-1 (A) and VCAM-1 (B) genes. HMECs were transiently transfected with ICAM-1 (A) and VCAM-1 (B) luciferase vectors into HMECs and incubated with or without visfatin (100 ng/ml) for 6 h. Data is the mean ± SE of triplicate experiments relative to the luciferase light units in untreated cells (set at 100%). *$P<0.01$ compared to untreated. C, HMECs were incubated with or without visfatin (500 ng/ml) in the presence or absence of 100 μM PDTC for 4 h. Total mRNAs were isolated, and RT-PCR analysis was performed using specific primers to human ICAM-1 and VCAM-1. β-actin served as an internal control.

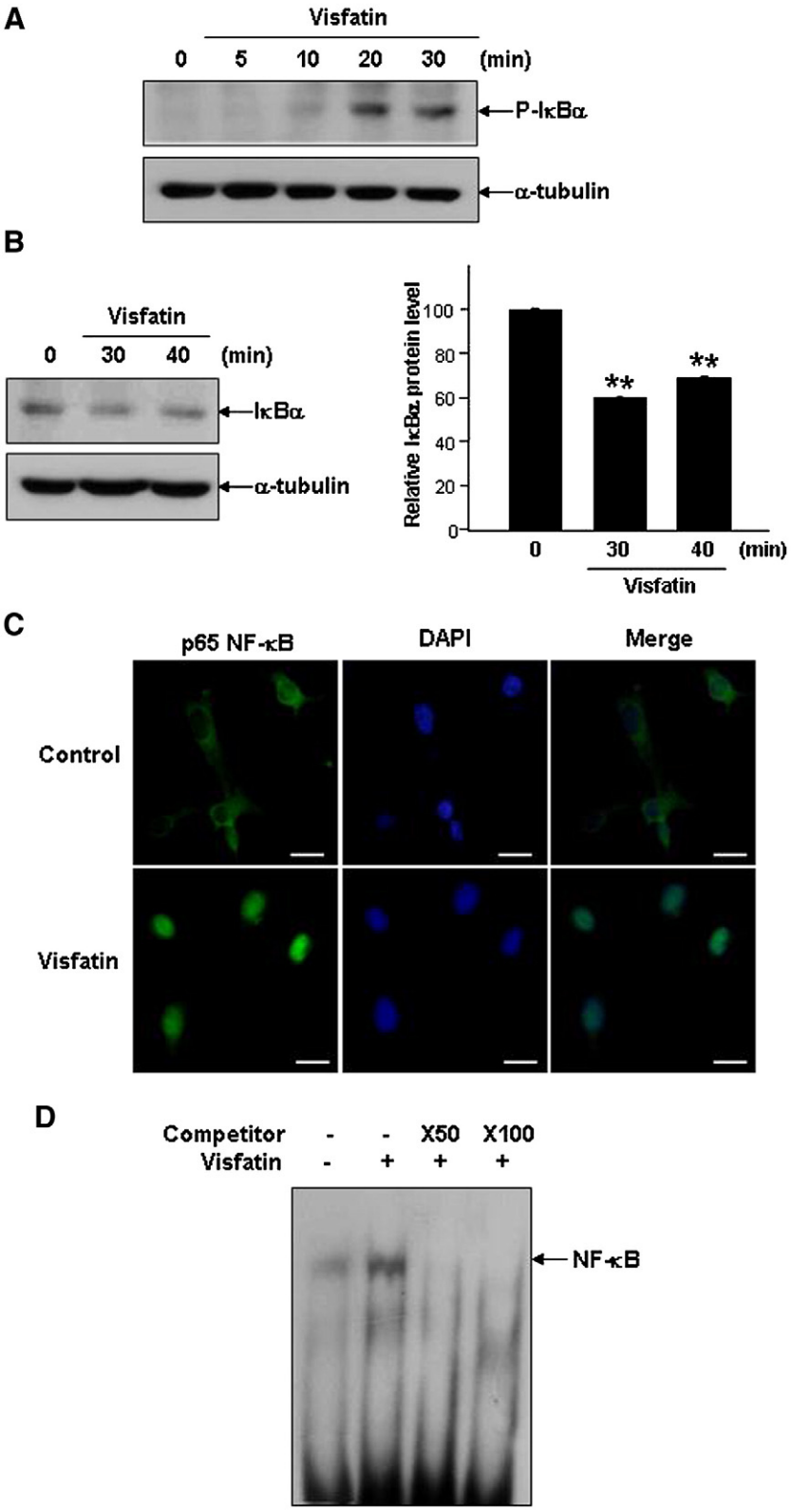


Fig. 4. Visfatin induces IκBα phosphorylation, NF-κB nuclear translocation, and NF-κB DNA binding activity. A, B, HMECs were incubated with or without visfatin (500 ng/ml) for the indicated times. Western blots were probed with anti-phospho-IκBα (A) and anti-IκBα (B) antibodies. α-tubulin served as the loading control. $^{**}P<0.05$ compared to untreated. C, HMECs was incubated with or without visfatin (500 ng/ml) for 30 min and subjected to immunocytochemical analysis of p65 localization as described in "Materials and methods". Cells were stained with NF-κB p65 (green) and nuclei were revealed with DAPI (blue). Bar=20 μm. D, NF-κB binding activities in nuclear extracts were measured by EMSA. HMECs were incubated with visfatin (500 ng/ml, lanes 2, 3 and 4) for 30 min. Nuclear extracts from these cells were incubated with biotin-labeled VCAM-1 NF-κB oligonucleotide. In the competition assay, 50× (lane 3) and 100× (lane 4) excess unlabeled probe were added to the reaction mixtures.

consensus sequence in HMECs (Fig. 4D, lane 2), which was abrogated by addition of excess unlabeled NF-κB consensus oligodeoxynucleotide (Fig. 4D, lanes 3 and 4). Thus, visfatin promotes NF-κB activation in endothelial cells.

3.5. Visfatin-induced CAM expression and NF-κB activation are associated with ROS production: involvement of NAD(P)H oxidase

Reactive oxygen species (ROS) produced by cytokines, growth factors, and vasoactive agents contribute to the intracellular signaling cascades associated with inflammatory responses [27,28]. ROS induce NF-κB activation by modifying the activity of one or more of the kinase enzymes in the NF-κB activation cascades [29]. Therefore, we investigated whether visfatin could induce ROS generation in HMECs. The fluorogenic probe, dihydroethidium (DHE), was used to assess the effect of visfatin on ROS generation. Also the thiol reducing agent *N*-acetyl-L-cysteine (NAC) is known as a direct scavenger of ROS and commonly used in studies to establish the role of ROS in gene expression [30]. As shown in Fig. 5A, visfatin significantly increased ROS generation, and visfatin-induced DHE fluorescence was abolished in the presence of NAC. We then examined the effect of NAC on the visfatin-induced ICAM-1 and VCAM-1 gene expression. The pretreatment of HMECs with NAC inhibited visfatin-induced ICAM-1 and VCAM-1 mRNA expression (Fig. 5B). We further investigated whether NAC down-regulates visfatin-induced NF-κB activation, leading to the decrease in the expression of ICAM-1 and VCAM-1. NAC also significantly suppressed visfatin-induced phosphorylation of IκBα in HMECs (Fig. 5C) and visfatin-induced NF-κB binding activity (Fig. 5D). Thus, visfatin mediates NF-κB activation and CAMs induction via ROS-dependent mechanism. And to determine the main enzymatic source of visfatin-induced ROS, we pretreated HMECs with inhibitors of different enzymatic sources of ROS. Pretreatment of HMECs with NAD(P)H oxidase inhibitor (DPI) significantly inhibited visfatin-induced increases in ICAM-1 and VCAM-1 mRNA expression (Fig. 5E). And NAD(P)H oxidase activity was examined by the measurement of lucigenin chemiluminescence. As shown in Fig. 5F, visfatin increased NAD(P)H oxidase activity, which was blocked by DPI, indicating that visfatin-induced ROS production occurs, at least in part, through NAD(P)H oxidase activation.

4. Discussion

In the present study, we demonstrated that visfatin induces ICAM-1 and VCAM-1 expression through ROS-dependent NF-κB activation in HMECs on the basis of the following evidence: (1) Visfatin induced adhesion of leukocytes to endothelial cells *in vitro* and *in vivo*, which correlated with ICAM-1 and VCAM-1 induction; (2) Visfatin-induced CAM expression required the activation of NF-κB; and (3) ROS were responsible for both visfatin-induced ICAM-1 and VCAM-1 expression and visfatin-stimulated activation of NF-κB.

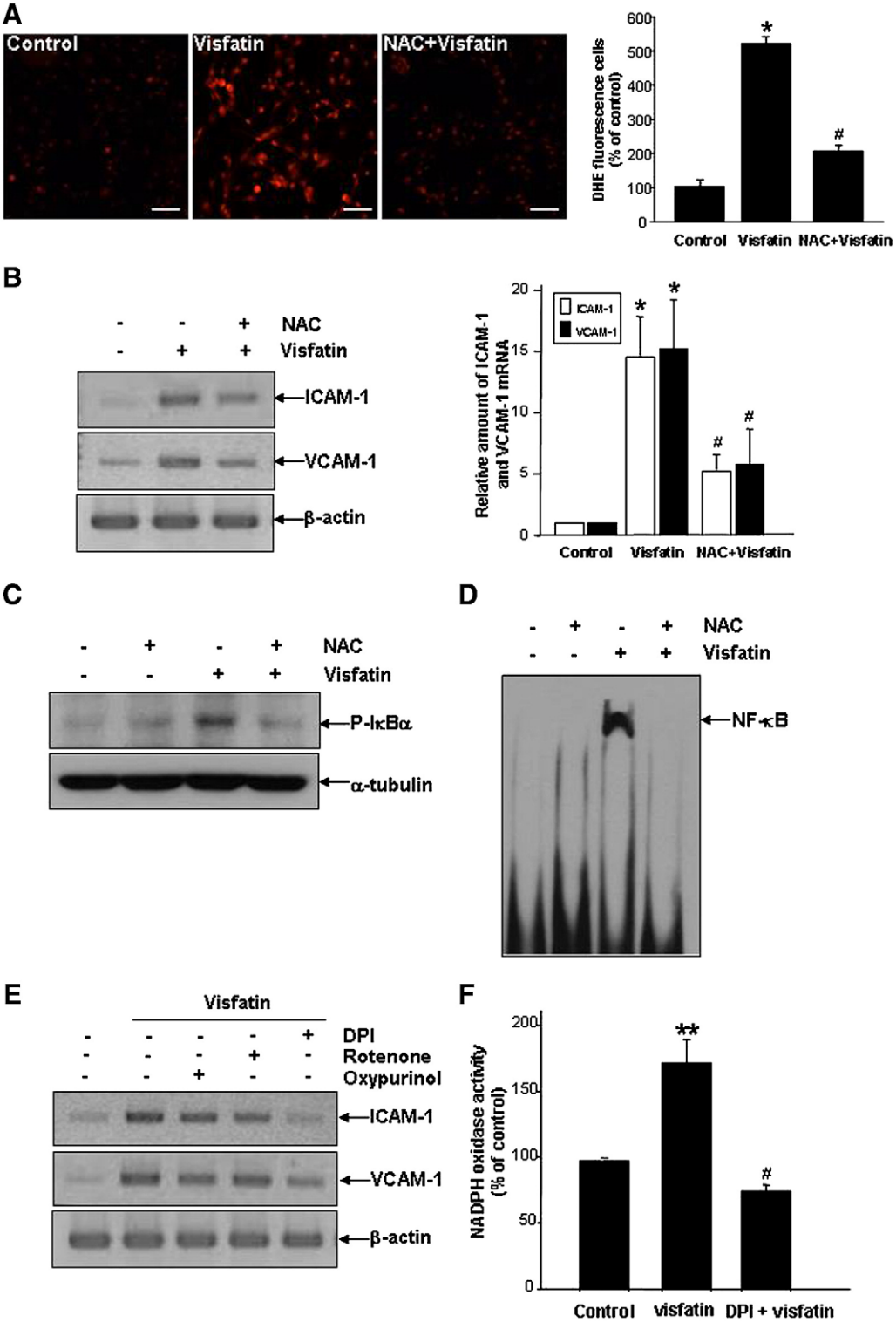
Obesity, characterized by an excess accumulation of body fat, is a significant risk factor for cardiovascular diseases such as atherosclerosis and hypertension [31,32]. Atherosclerosis is an inflammatory process that begins with systemic endothelial dysfunction, a pivotal event in the early pathogenesis of atherosclerotic lesion formation [4,33]. The molecular mechanisms linking obesity and atherosclerosis have yet to be fully elucidated, but seem to involve fat-derived adipokines affecting endothelial function and vascular homeostasis [7].

Adipokines such as leptin and resistin regulate the endothelial expression of cell adhesion molecules and chemoattractant chemokines, one of the early important steps in atherosclerosis [34,35]. These also serve as the cellular mediator of endothelial dysfunction and metabolic syndrome of insulin resistance, which exerts proatherogenic effects such as lipid abnormalities, platelet aggregation and hypertension. In particular, leptin augmented thrombus and atheroma formation by promoting vascular inflammation, arterial hypertension, endothelial free radical overproduction, and cholesterol accumulation under hyperglycemia [36–39]. Recently, it has been reported that the expression of visfatin is high at plaque rupture sites in patients with coronary artery disease [22]. We showed that visfatin accelerates monocyte adhesion to endothelial cells by up-regulating adhesion molecules in vascular endothelial cells (Figs. 1 and 2), suggesting a possible role for visfatin in the development of atherosclerosis. Of course, further studies are necessary to clarify the atherogenic effect of visfatin and its underlying molecular mechanism in the obesity-linked insulin resistance, diabetes, and atherosclerosis.

The NF-κB transcription factor plays a significant role in regulating proinflammatory genes in response to inflammatory stimuli [40,41]. Kinases in the PI3K/Akt and MAPK cascades regulate the NF-κB-dependent pathway [42,43]. In this study,

Fig. 5. Involvement of ROS in visfatin-induced ICAM-1 and VCAM-1 expression and NF-κB activation. HMECs were incubated with or without visfatin (500 ng/ml) in the presence or absence of inhibitors for 30 min (A, C, D and F) and 4 h (B and E). A, ROS levels were detected using the fluorogenic probe DHE as detailed in the "Materials and methods". Bar = 100 μm. Data are mean ± SE of triplicate experiments relative to the DHE fluorescence units in untreated cells (set at 100%). $^{*}P<0.01$ compared to control; $^{\#}P<0.01$ compared to visfatin. B, HMECs were pretreated with or without NAC (5 mM) for 30 min. Total RNAs were isolated and then analyzed by RT-PCR using specific primers to human ICAM-1 and VCAM-1. β-actin served as an internal control (left). Using real-time RT-PCR, we also quantified the expression levels of ICAM-1 and VCAM-1 mRNA. Culture medium was supplemented with 10 μg/ml polymyxin B. The expression level of control (untreated) was set to 1, and the values are normalized to β-actin mRNA levels (right). $^{*}P<0.01$ compared to control; $^{\#}P<0.01$ compared to visfatin. C, HMECs were pretreated with or without NAC (5 mM) for 30 min. Western blots were probed with anti-phospho-IκBα antibodies. α-tubulin served as loading control. D, Nuclear extracts were assayed for NF-κB binding activities by EMSA. Culture medium was supplemented with 10 μg/ml polymyxin B. E, HMECs were pretreated with or without the NAD(P)H oxidase inhibitor, DPI (10 μM), the xanthine oxidase inhibitor, Oxypurinol (10 μM), or the mitochondrial NADH dehydrogenase inhibitor, rotenone (2 μM) for 30 min. Total mRNAs were isolated, and RT-PCR analysis was performed using specific primers to human ICAM-1 and VCAM-1. β-actin served as an internal control. F, HMECs were pretreated with or without DPI (10 μM) for 30 min and then NAD(P)H oxidase activity was measured by luminescence assay in 50 nM phosphate buffer containing NAD(P)H as a substrate. $^{**}P<0.05$ compared to control; $^{\#}P<0.01$ compared to visfatin.

we demonstrated that visfatin induces NF-κB activation, which in turn mediates NF-κB-dependent expression of the endothelial cell adhesion molecules, ICAM-1 and VCAM-1 (Fig. 4). Indeed, visfatin up-regulates cytokine production via the p38MAPK pathway and NF-κB p65 (RelA) DNA binding activity in human leukocytes [9]. We previously showed that visfatin stimulates extracellular signal regulated kinases 1/2 (ERK1/2) and slightly activates Akt in endothelial cells [11]. On the basis of these findings, visfatin may up-regulate NF-κB activity via activation of signal cascades such as MAPK and PI3K/Akt pathway. However, we cannot exclude the possibility that visfatin may work secondary to the release of other inflammatory cytokines or proteins from endothelial cells, resulting in NF-κB activation. These possibilities are currently under investigation.



Visfatin binds to and activates the insulin receptor via a distinct binding site from insulin, which stimulates glucose uptake in adipocytes [8]. In addition to the regulation of glucose homeostasis, visfatin modulates immunomodulatory and inflammatory processes [9,10]. Visfatin also increases cytokine levels in monocytes, whereas insulin does not, suggesting the presence of an unidentified visfatin receptor [9,44]. In our preliminary study, we checked whether the up-regulation of CAMs induced by visfatin is dependent on insulin receptor activation. Thus, we treated HMECs with HNMPA-$(AM)_3$ (hydroxy-2-naphthalenylmethylphosphonic acid tris-acetoxy-methyl ester), a specific inhibitor of insulin receptor tyrosine kinase [45]. HNMPA-$(AM)_3$ had little effect on visfatin-stimulated ICAM-1 and VCAM-1 mRNA levels and visfatin-induced NF-κB DNA binding activity (data not shown). Although further studies are needed, these results suggest that the effects of visfatin on CAMs expression may be mediated by its own unidentified receptor.

Oxidative stress is an important determinant of endothelial dysfunction and vascular injury [46]. ROS influence a number of cellular responses by turning on several intracellular signaling cascades in vascular cells, which contribute to altered vascular tone, vascular inflammation, and vascular remodeling, thus leading to the progression of vascular diseases [47]. We also showed that visfatin stimulates ROS generation in endothelial cells, and antioxidants blocked visfatin-induced NF-κB activation and CAM expression (Fig. 5). Based on these observations, visfatin may be able to accelerate obesity-related vascular diseases through ROS overproduction by inducing vascular damage. NAD(P)H oxidase is a major source of ROS generation in vascular endothelial cells, and critical in the regulation of oxidative stress in the vasculature [48]. Dysregulated NAD(P)H oxidase activity is associated with marked endothelial dysfunction in various states of vascular diseases [49]. We showed that visfatin-induced CAM expression is significantly regulated by NAD(P)H oxidase inhibitor, and visfatin enhances NAD(P)H-dependent ROS production in endothelial cells. Of course, future studies will be needed to determine the exact molecular mechanisms underlying visfatin-induced endothelial NAD(P)H oxidase activation and the potential roles of endothelial NAD(P)H oxidase in obesity-related vascular dysfunction.

Precise understanding of pathophysiology and molecular actions of visfatin will lead to the discovery of effective therapeutic intervention. Development of pharmacological visfatin antagonists and novel manipulations which inhibit visfatin signaling may be promising strategies to attenuate the proinflammatory effects of visfatin on endothelial cells and ultimately to reduce the progression of obesity-related vascular diseases.

In conclusion, the present study demonstrated for the first time that a novel visceral adipokine, visfatin, has direct proinflammatory effects on vascular endothelial cells, which promote the expression of endothelial cell adhesion molecules through ROS-dependent NF-κB activation. Our findings provide a potential role for visfatin in the pathogenesis of endothelial dysfunction and a variety of vascular inflammatory disorders. This knowledge may contribute to the development of new therapies for obesity-related vascular diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2008.01.004.

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