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Biochemical and Biophysical Research Communications 357 (2007) 150–156
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Visfatin promotes angiogenesis by activation of extracellular signal-regulated kinase 1/2

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Received 16 March 2007
Available online 28 March 2007

Abstract

Adipose tissue is highly vascularized and requires the angiogenic properties for its mass growth. Visfatin has been recently characterized as a novel adipokine, which is preferentially produced by adipose tissue. In this study, we report that visfatin potently stimulates *in vivo* neovascularization in chick chorioallantoic membrane and mouse Matrigel plug. We also demonstrate that visfatin activates migration, invasion, and tube formation in human umbilical vein endothelial cells (HUVECs). Moreover, visfatin evokes activation of the extracellular signal-regulated kinase 1/2 (ERK1/2) in endothelial cells, which is closely linked to angiogenesis. Inhibition of ERK activation markedly decreases visfatin-induced tube formation of HUVECs and visfatin-stimulated endothelial cell sprouting from rat aortic rings. Taken together, these results demonstrate that visfatin promotes angiogenesis via activation of mitogen-activated protein kinase ERK-dependent pathway and suggest that visfatin may play important roles in various pathophysiological angiogenesis including adipose tissue angiogenesis.

Keywords: Visfatin; Vascular endothelial cells; Angiogenesis; ERK

Adipokines are biologically active polypeptide growth factors and cytokines that appear to be mainly produced by adipose tissue [1]. They exhibit extensive effects in the immune and metabolic system through paracrine and endocrine fashions [2]. A number of recent studies have explored the effects of adipokines on endothelial function and pathogenesis of vascular disorders [3,4].

Angiogenesis, the formation of new blood vessels emerging from pre-existing endothelial vasculature, is an integral component of the pathogenesis of various disorders, such as tumor growth, diabetic retinopathy, chronic inflammation, and obesity [5]. Obesity is characterized by an excess accumulation of body fat [6]. This process requires the formation of new capillaries for functional mass growth [7,8]. Some of adipokines such as leptin, resistin, and adiponectin are potentially involved in the regulation of neovascularization [9–11]. Aberrant angiogenesis is often associated with obesity-related vascular diseases including atherosclerosis, diabetes, and hypertension [4,5].

Visfatin (pre-B-cell colony-enhancing factor, PBEF) has been originally isolated from peripheral blood lymphocytes and recently characterized as a novel adipokine exerting insulin-mimetic effects on various insulin-sensitive tissues

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0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.03.105

[12,13]. Recently, several reports showed that visfatin (PBEF) is involved in thrombin-induced lung endothelial barrier dysregulation and promotes the efficient acquisition of a mature smooth muscle cell phenotype that is essential for remodeling of blood vessel, indicating a possible role in vascular homeostasis [14,15]. We previously reported that human visfatin gene is transcriptionally activated in response to hypoxia which can mainly trigger molecular determinants involved in different steps of angiogenesis [16].

In this present study, we investigated the angiogenic activities of visfatin and its underlying molecular mechanism in endothelial cells. Our results clearly showed that visfatin markedly promotes *in vivo* new blood vessel formation and enhances the migration, transinvasion, and tube formation of endothelial cells. Furthermore, we demonstrated that the angiogenic effect of visfatin is mediated by the activation of ERK in endothelial cells.

Materials and methods

Reagents and recombinant proteins. Antibodies for phospho-ERK (Thr-202/Tyr-204) and ERK were obtained from New England Biolabs. VEGF and U0126 were purchased from Upstate Biotechnology and Promega, respectively. The full-length human visfatin expression vector was constructed by PCR and subcloned into pET vector (Novagen). For production of His fusion protein, pET-visfatin was transformed and His-visfatin protein was purified on Talon metal affinity column (CLONTECH). This protein was dialyzed using Sephadex G-25 column (Amersham Pharmacia Biotech).

Chorioallantoic membrane (CAM) assay. Thermanox coverslips were loaded with recombinant visfatin, which, after air-drying, were then applied to the CAM surface of 9-day-old chick embryos. Three days later, an appropriate volume of 10% fat emulsion (10% Intralipose) was injected into the 12-day-old embryo chorioallantois, and angiogenesis observed 12× magnification under a microscope

In vivo Matrigel plug assay. C57/BL6 mice were injected subcutaneously with 0.5 ml of Matrigel (BD Bioscience) containing the indicated amount of visfatin with heparin (50 U/ml). After 7 days, mice were killed, and the Matrigel plugs were recovered, fixed with 3.7% formaldehyde/phosphate-buffered saline, and embedded in paraffin. Part of each plug was fixed, sectioned, and either stained with hematoxylin/eosin or immunostained with antibody for PECAM-1, a marker for endothelial cells. Hemoglobin was measured, using the Drabkin method, for the quantification of blood vessel formation.

Intravital fluorescence microscopy. A titanium abdominal wall window was implanted over the liver and sutured between the skin and abdominal wall of male BALB/c mice. The glass coverslip was placed on the window and fixed with the snap ring. After the mice were stabilized, 100 μl of Matrigel was layered in the presence or absence of visfatin at the abdominal wall. On day 5, high-molecular-weight (500,000) dextran labeled with FITC (Sigma–Aldrich) was administered intravenously (0.05 mg/ gm of bodyweight). Microvasculature was episcopically visualized under a fluorescence intravital microscope. Real-time imaging was recorded at 30 frames/s using the Cascade 650 CCD camera (RoperScientific).

Cell culture. Primary human umbilical vein endothelial cells (HUVECs) (passage 5–8) were purchased 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. A human microvascular endothelial cell line (HMEC-1) was obtained from CDC (Atlanta, GA). These cells were grown on uncoated plastic in medium MCDB 131 supplemented with EGF (10 ng/mL), hydrocortisone (1 μg/ml), and 10% FBS.

Tube formation assay. Two hundred and fifty microliters of Matrigel was pipetted onto 24-well culture plates and polymerized for 30 min at 37 °C. HUVECs were seeded onto the surface of the Matrigel, and visfatin then added and the plates incubated for 10 or 20 h at 37 °C. Morphological changes of the cells were observed under a microscope and photographed.

Wounding migration assay. At 90% confluence, the endothelial monolayers in 60 mm culture dishes were marked with an injury line, and a 2 mm in width wounded with a sterile razor blade. Plates were rinsed with serum-free medium to remove cellular debris, and fresh medium containing visfatin and 1 mM thymidine then added. HUVECs were allowed to migrate for 16 h, rinsed with serum-free medium, and photographs then were taken using an inverted microscope.

Chemoinvasion assay. The lower and upper sides of 8 μm porosity polycarbonate filters were coated with 0.5 mg/ml type I collagen and 0.5 mg/ml Matrigel, respectively. The lower compartment contained visfatin-treated medium with HUVECs placed in the upper part of a Transwell plate. Cell invasion was determined by counting whole cell numbers on a single filter using optical microscopy at 40× magnification.

Aortic ring sprouting assay. Aorta was excised from 6-week-old male Sprague–Dawley rats and fibroadipose tissue was removed. The aorta was sectioned into 1-mm thickness, and then aorta rings were placed on Matrigel-coated wells, covered with an additional 50 μl Matrigel, and allowed to gel for 30 min at 37 °C. Visfatin was added to the wells in final volume of 200 μl of human endothelial serum-free media. On day 5, microvessel outgrowth was photographed under a phase contrast microscope. Sprouting scores classified from 0 (least positive) to 6 (most positive). Data shown are means ± SD from three different experiments conducted in triplicate. $^{*}P < 0.05$ compared to control. $^{**}P < 0.05$ compared to visfatin only (Supplementary data shown).

Results

Visfatin induces angiogenesis in vivo

To examine whether visfatin has an angiogenic activity, a CAM assay was first performed. Application of the coverslip containing visfatin resulted in the radial development of new blood vessels toward the coverslip in a wheel pattern (Fig. 1A). Using a control thermanox coverslip, containing vehicle alone, no growth of new blood vessels was observed. The angiogenic activity of visfatin (1 μg/egg) was 57.7 ± 5.8% ($n = 18$) (Fig. 1B). The *in vivo* angiogenic activity of visfatin was further evaluated by an established *in vivo* angiogenesis model, the mouse Matrigel plug assay, and observed under a fluorescence intravital microscope. Visfatin produced more neovessels within gels than Matrigel alone. The vascular density and the number of mature vascular structures were significantly increased by visfatin (Fig. 2A, intravital). On histological examination, control plug in which Matrigel was injected with heparin alone showed few vessels, but visfatin enhanced abundant vessel development with PECAM-1-positive endothelial cells inside the plugs (Fig. 2A, H-E and PECAM-1). To quantify the functional vasculature, the hemoglobin contents of the Matrigel plugs were measured. The new vessels were abundantly filled with intact red blood cells, which indicate the formation of a functional vasculature inside the Matrigel; the hemoglobin content of visfatin-containing gel was significantly higher than that of negative control

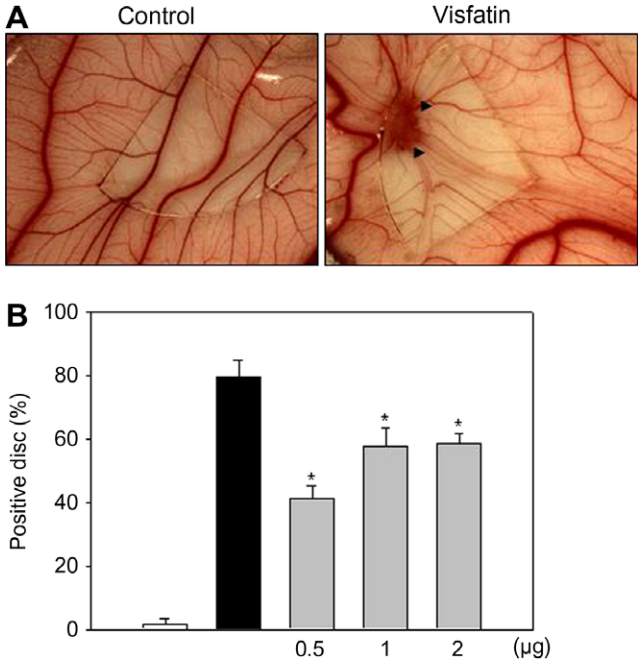

Fig. 1. Angiogenic activity of visfatin on the chick CAM. (A) The chick chorioallantoic membrane surfaces of 9-day-old chick embryos were treated with thermanox coverslips in the absence or presence of visfatin protein. Three days later, angiogenesis was observed under a microscope. A vascular zone is indicated by the arrowheads. (B) Angiogenic responses were scored as positive when visfatin-treated CAM showed a vascular zone, which had many vessels compared with the control, and calculated by the percentage of positive eggs. White bar, empty coverslip as negative control; black bar, 0.1 μg PMA as positive control; gray bars, visfatin at indicated concentrations. Independent experiments were repeated 3 times, and each value indicates the means ± SE of more than 15 eggs. $^{*}P < 0.05$ compared to control.

gel (Fig. 2B). Taken together, these results indicate that visfatin has a potent angiogenic activity *in vivo*.

Visfatin stimulates migration, invasion, and tube formation of endothelial cells

To explore the angiogenic effects of visfatin, *in vitro* angiogenesis assays were conducted using HUVECs. The migration of endothelial cells is a critical, initiating event in the formation of new blood vessels and the repair of injured vessels. Confluent cultures of HUVECs were wounded with a razor blade, with migration measured as the number of cells moving into the wounded area. The migration assay showed that the mobility of HUVECs was significantly enhanced under treatment with visfatin (Fig. 3A). As another important property of angiogenesis, migrating endothelial cells must break and transverse through their basement membrane to form new blood vessels. The HUVECs were placed in the polycarbonate filters of Matrigel-coated Transwell and allowed to invade in the presence of visfatin. As shown in Fig. 3B, visfatin significantly increased the invasiveness of the HUVECs. To determine the effect of visfatin on the tubular formation of HUVECs, visfatin was treated to HUVECs seeded on Matrigel beds. As shown in Fig. 3C, various concentrations of visfatin stimulated the capillary network formation of endothelial cells, resulting in robust and elongated tube-like structures. These results further indicated that visfatin has a potent angiogenic action via the stimulation of various angiogenic steps.

Visfatin stimulates the activation of ERKs in endothelial cells

Recent studies have shown that activation of ERKs is closely involved in the migration or tubular-like formation of endothelial cells and induced by various angiogenic factors [17–19]. Therefore, whether visfatin can stimulate ERKs in endothelial cells was investigated. Vascular endothelial cells were exposed to visfatin, and the activation of ERK1/2 was analyzed by Western blot analysis using antibody directed against the phosphorylated form of ERK1/2 (p44 ERK1 and p42 ERK2). As shown in Fig. 4A, visfatin induced ERK activation of cells in time-dependent manners. Pretreatment of cells with the specific inhibitor of MAPK/ERK kinase, U0126, blocked the ability of visfatin to activate ERK1/2 (Fig. 4B). To determine the role of ERK activity on the stimulation of new blood vessel formation by visfatin, we performed tubular formation assay in which cells were pretreated with U0126 prior to exposure to visfatin. Pretreatment of cells with U0126 blocked the visfatin-induced tube formation of endothelial cells (Fig. 4C). Treatment with U0126 also effectively inhibited visfatin-enhanced invasive activity of vascular endothelial cells (data not shown). Using aorta model of *ex vivo* angiogenesis, we confirmed the involvement of ERK in visfatin-induced angiogenesis. Rat aortic rings were placed in Matrigel and incubated with visfatin in the presence or absence of U0126. As shown in Fig. 4D and Fig. 4D supplementary data, visfatin increased microvessel sprouting from the adventitia of aortic rings. This microvessel outgrowth was inhibited by treatment with U0126, indicating ERK activity is required for the angiogenic response to visfatin.

Discussion

Visfatin (also known as pre-B cell colony-enhancing factor) was originally cloned as a cytokine-like growth factor, expressed by lymphocyte, enhancing the effects of IL-7 and stem cell factor on early stage B cells [12]. It is preferentially produced in visceral adipose tissue and recently known as a novel adipokine exerting insulin-mimetic effect on various insulin-sensitive tissues [13]. Although roles of typical adipokines on the vascular functions have been reported [3,4], the effect of recombinant visfatin on endothelial cells or vasculature has not been elucidated yet.

In this report, the *in vitro* and *in vivo* angiogenic activities of visfatin have been shown. Adipose tissue is closely related to angiogenesis. The growth potential of adipose tissue through adulthood requires the ability to recruit new capillaries. In this process, adipocytes secrete a

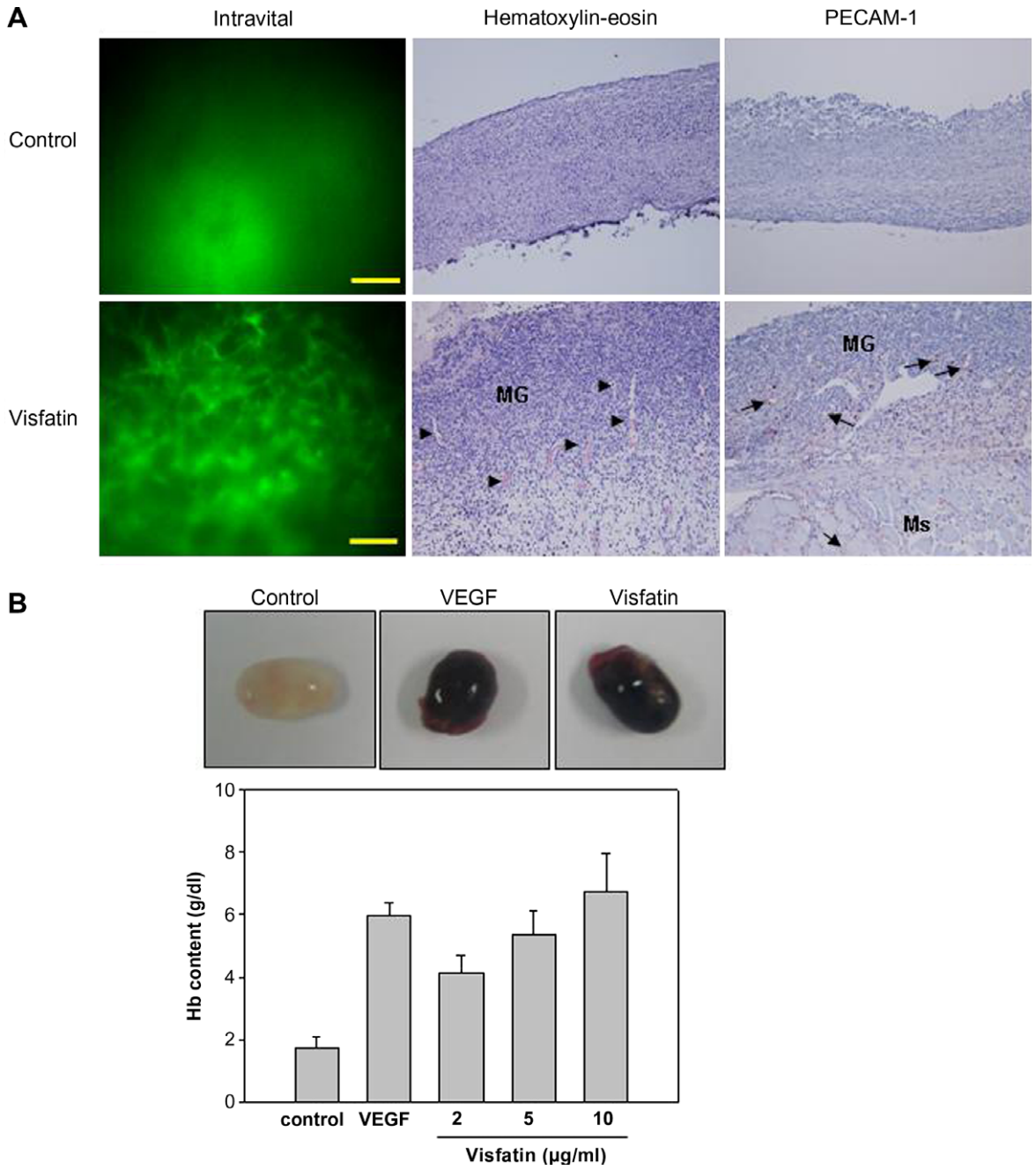

Fig. 2. Angiogenic activity of visfatin in mouse Matrigel plug. (A) The newly grown microvessels in Matrigel layer were visualized with *in vivo* intravital microscope in the presence or absence of visfatin (6 μg/ml). The Matrigel was collected and stained with hematoxylin–eosin and immunohistochemical procedure against PECAM-1. The vessel (arrowhead) in Matrigel (MG) and PECAM-1-positive endothelial cells (arrow) were found in Matrigel (MG) and muscle (Ms) layers, bar = 100 μm. (B) Matrigel plugs were photographed. VEGF (50 ng/ml) as positive control; visfatin (10 μg/ml). The concentration of hemoglobin was calculated from a parallel assay with a known amount of hemoglobin. Each value represents the mean of at least four animals, and similar results were obtained in two different experiments; bars, ± SE.

number of adipokines such as VEGF, HGF, leptin, and adiponectin which can function as angiogenic factors [9,11,20,21], leading to *in vitro* or *in vivo* neovascularization by paracrine or endocrine manner. Therefore, it can be expected that visfatin secreted by adipocytes can actually contribute to angiogenesis-dependent fat mass growth and coordinate angiogenesis with adipogenesis during *in vivo* adipose tissue expansion.

Obesity is a risk factor for cardiovascular diseases, namely atherosclerosis and hypertension, whose progress involves dysregulated angiogenesis [5,22]. Progressive angiogenesis in atherosclerotic lesions has been considered one of the causes of plaque expansion and vulnerability, and a risk of significant disease complications, such as plaque rupture and vascular thrombosis [23]. Several studies have demonstrated that some adipokines

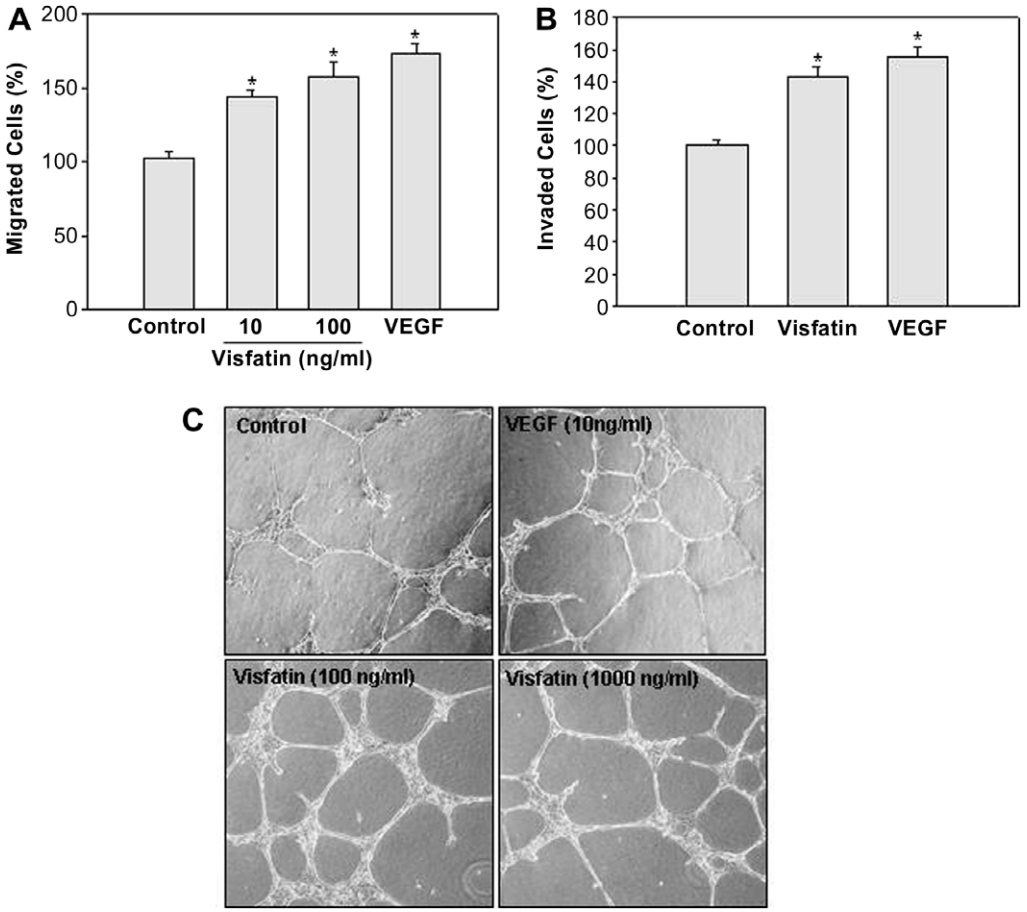

Fig. 3. Effect of visfatin on *in vitro* angiogenesis of HUVECs. (A) HUVECs were seeded on the gelatin-coated culture dish. At the 90% confluence, the monolayers were wounded with a razor blade. Wounded cells were incubated without or with visfatin (10 or 100 ng/ml) for 16 h. The number of HUVECs that moved beyond the reference line was counted. Data are means ± SD of triplicate determinations. (B) HUVECs in the absence or presence of the visfatin (100 ng/ml) were incubated on transwell chambers for 16 h. The numbers of invaded cells were counted. The data are expressed as percentage ± SE from three different experiments with duplicate. $^{*}P < 0.05$ compared to control. (C) HUVECs were incubated on the Matrigel without or with visfatin for 10 h. In these experiments, VEGF (10 ng/ml) was used as positive control.

can link between angiogenesis and atherosclerosis [3]. For example, leptin, one of typical adipokines, promotes the growth of atheromatous plaques through its effect on neovascularization [24,25]. Interestingly, it has recently been reported that visfatin is highly expressed at the site of plaque rupture in patient with coronary artery disease [26]. Based on these reports, we suggest a possible role of visfatin in the development of atherosclerosis, which is based on abnormal neovascularization, leading to coronary vascular disease.

Insulin receptor is known to be binding receptor of visfatin in a manner distinct from insulin [13]. However, the effects of visfatin are not restricted to glucose homeostasis. In many cases it has been suggested that the visfatin has proinflammatory and immunomodulating properties [27–29]. Moschen et al. reported that visfatin leads to increased levels of cytokine, whereas insulin does not show cytokine-inducing effects, suggesting that induction of cytokines by visfatin may be mediated by engagement of unidentified receptor [29]. Of course, further investigations will be necessary to clarify if visfain has its own receptor or works through insulin receptor in visfatin-induced angiogenesis.

Our data provided in this study that visfatin stimulates the activation of ERKs in endothelial cells (Fig. 4). It has been reported that visfatin induces the phosphorylation of Akt in adipocytes, myocytes, and hepatocytes, resembling the effects of insulin [13]. We also observed that the treatment of visfatin in HUVECs slightly activates Akt (data not shown). A recent study showed that inhibition of p38MAPK abrogates visfatin-induced cytokine production in monocytes [28]. From these data, Akt signal or another member of the MAPK family pathway may in part contribute to the angiogenic process triggered by visfatin. These possibilities are under investigation.

We demonstrated that recombinant visfatin directly stimulates endothelial cells, leading to *in vitro* angiogenic activities. In many cases it has been suggested that the *in vivo* effect of angiogenic factors may be secondary to the release of angiogenic molecules from adjacent nonendothelial cells [30]. Visfatin has been considered a new proinflammatory adipokine which may be able to stimulate

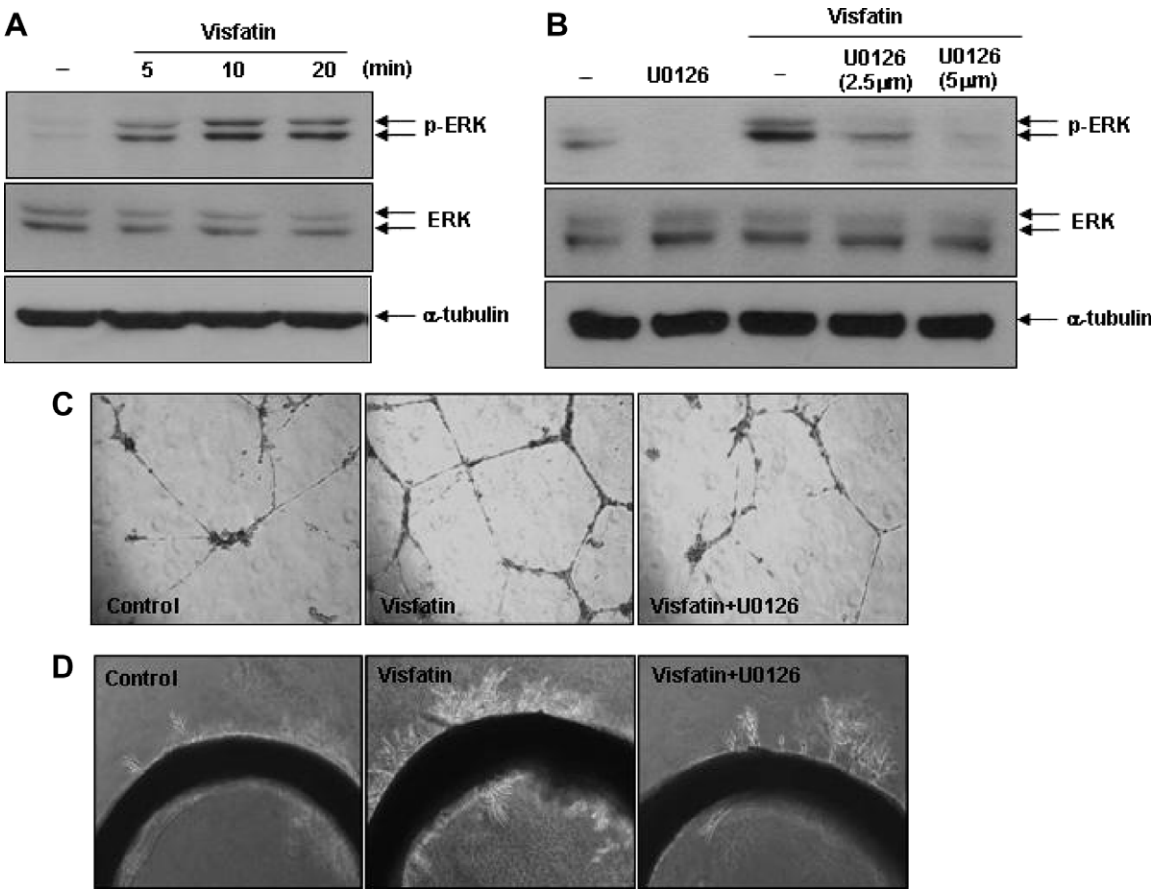

Fig. 4. Visfatin-induced ERKs activation of vascular endothelial cells. (A) Endothelial cells (500 ng/ml) were stimulated with visfatin at the indicated times. Phosphorylated forms of ERKs in whole cell extracts were detected using phosphoERK-specific antibody. The membranes were stripped and reprobed with an antibody against ERK. (B) Cells were preincubated with U0126 for 30 min prior to stimulation with control or visfatin for 10 min. ERK activation was measured by Western blot analysis for pERK. (C) HUVECs were incubated at 37 °C on Matrigel without (control) or with visfatin or visfatin + U0126. After 20 h, photographs were taken (40×). (D) Rat aortic rings were embedded in Matrigel and cultured for 5 days with visfatin in the presence or absence of U0126 (10 μM). Pictures are the representative photographs for endothelial cell sprouts formed from the aortic ring segments.

monocytes, macrophages, platelets, mast cells, and other leukocytes to release a myriad of angiogenic factors [5]. Therefore, we cannot exclude the possibility that *in vivo* angiogenic activity of visfatin in our study, in part, may be due to angiogenic factors and cytokines secreted from nonendothelial cells.

In conclusion, we have demonstrated for the first time that visfatin exhibits angiogenic actions via the activation of ERK1/2. Our data may provide evidence that an important role of a newly characterized adipokine, visfatin, in the pathogenesis of angiogenesis-related vascular disorders, which may be linked to obesity, cardiovascular disease, and solid tumors, as well as a novel approach to target the development of new therapeutic strategies.

Acknowledgments

This work was supported by the research grant from a Vascular System Research Center grant from KOSEF, Korea Research Foundation Grant funded by Korea Government (MOEHRD, basic Research Promotion Fund) (KRF-2005-204-C00051), Medical Research Institute Grant (2005-07), Pusan National University (to M.-K. Bae), and MRC program of MOST/KOSEF (R13-2005-009) (to S.-K. Bae).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.03.105.

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