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Vaspin Can Not Inhibit TNF-α-Induced Inflammation of Human Umbilical Vein Endothelial Cells

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ABSTRACT. Visceral adipose tissue-derived serine protease inhibitor (vaspin) has been recently identified as an adipocytokine in a rat model of type 2 diabetes. Adipocytokines may directly influence the function of endothelial cells (ECs) and modulate inflammatory states. We therefore assessed the effects of vaspin on basal and TNF-α-stimulated human umbilical vein ECs. Vaspin (10–100 *n*g/m*l*, 24 hr) had no effects on both basal ECs morphology and TNF-α-induced (10 *n*g/m*l*, 24 hr) morphological damages. Vaspin did not inhibit the TNF-α (20 min) activation of JNK, p38 and NF-κB, but only slightly inhibited Akt. Furthermore, vaspin did not decrease the TNF-α (24 hr) induction of vascular cell adhesion molecule-1, intercellular adhesion molecule-1, endothelial selectin, and cyclooxygenase-2 protein expression as well as monocyte chemotactic protein-1, tissue factor, and plasmogen activator inhibitor-1 mRNA expression. The present results indicate that vaspin has no effects on normal ECs, and can not prevent TNF-α-induced inflammatory injury.
KEY WORDS: adipokine, endothelial cell, inflammation, signal transduction.

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Due to the worldwide rise in obesity during the past decades, adipose tissue has attracted enormous scientific interests. The tissue is now regarded as an active endocrine organ that releases a large number of adipokines modulating inflammation and other various processes [9].

As one of those potentially important molecules, vaspin (visceral adipose tissue-derived serine protease inhibitor) was initially identified as a novel adipokine with an insulin-sensitizing effect, from visceral white adipose tissues of Otsuka Long-Evans Tokushima fatty (OLETF) rat, an animal model of abdominal obesity with type 2 diabetes [11]. The serum levels of vaspin increase at the peak of obesity and insulin resistance whereas they decrease with the worsening of diabetes in OLETF rats. In humans, the correlations between serum vaspin levels and markers of insulin sensitivity, glucose metabolism, and obesity are controversial. Klöting *et al.* concluded that human vaspin mRNA expression could be associated with parameters of obesity, insulin resistance, and glucose metabolism [18]. Gulcelik *et al.* also found that serum vaspin levels were correlated with insulin resistance in diabetic patients [10]. In contrast, Seeger *et al.* failed to find this correlation [25]. Youn *et al.* found an association between vaspin serum levels and body mass index and insulin sensitivity but could not confirm this correlation in patients with type 2 diabetes [36]. In addition, administration of vaspin to obese ICR mice fed with high fat and sucrose chow suppressed the expressions of TNF-α, leptin, and resistin in mesenteric and subdermal white adipose tissues [11]. On the other hand, vaspin is also a member of serine protease inhibitor family, exhibiting approximately 40% homology with α1-antitrypsin [11]. Although vaspin is mainly confined to the adipocytes, it may have an effect on the endothelial cells in an analogous manner to another proteinase inhibitor, plasmogen activator inhibitor-1 (PAI-1), which is derived from mesenteric fat and can inhibit endothelial cell migration and angiogenic branching [22, 27].

Obesity predisposes to cardiovascular diseases, such as coronary heart disease, cerebrovascular disease, and peripheral vascular disease [3]. And impaired endothelial function is an independent predictor of cardiovascular disease [26]. Besides a variety of inflammation-related molecules, adipokines such as leptin [30], adiponectin [5] and resistin [28], can influence the function of endothelial cells and modulate inflammatory states [8, 19, 20, 24].

Taken together, we hypothesized that vaspin could affect vascular endothelial cells, especially in terms of inflammation. To our knowledge, the relationship between vaspin and the inflammation of endothelial cells is unknown. Therefore, in this study we investigated the effects of vaspin on basal and TNF-α-stimulated human umbilical vein endothelial cells (HUVECs).

MATERIALS AND METHODS

Materials: Reagent sources were as follows: recombinant human vaspin (PeproTech, Inc., Rocky Hill, NJ, U.S.A.); tumor necrosis factor-alpha (TNF-α) (Hoffmann-La Roche Ltd, Basel, Switzerland); and Superscript II Reverse Transcriptase (RT) (Invitrogen, Carlsbad, CA, U.S.A.). Antibody sources were as follows: phospho-Akt, -p38, -NF-κB p65 (Ser536) (Cell Signaling, Beverly, MA, U.S.A.); vascular cell adhesion molecule-1 (VCAM-1), cyclooxygenase (COX)-2, endothelial nitric oxide (NO) synthase (eNOS), IκB-α, and total NF-κB p65 (Santa Cruz Biotech, Santa Cruz, CA, U.S.A.); total actin (Sigma-Aldrich, St. Louis,

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MO, U.S.A.); endothelial selectin (e-selectin) and intercellular adhesion molecule-1 (ICAM-1) (R&D Systems, Inc., Minneapolis, MN, U.S.A.); and phospho-JNK (Promega, Madison, WI, U.S.A.).

Cell culture: HUVECs were purchased from Kurabo (Osaka, Japan) and cultured in Medium 200 supplemented with low serum growth supplement (LSGS; Cascade Biologics, Portland, OR, U.S.A.) as described previously [32–34]. Cells at passage 4 to 7 were used for experiments. HUVECs morphological changes were observed under light microscope (CKX31, Olympus, Tokyo, Japan) equipped with digital camera (SP-350, Olympus).

Western blotting: Western blotting was performed as described previously [32–35]. Protein lysates were obtained by homogenizing HUVECs with Triton-based lysis buffer (1% Triton X-100, 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na_3VO_4, 1 μg/m*l* leupeptin and 0.1% protease inhibitor mixture; Nacalai Tesque, Kyoto, Japan). Protein concentration was determined using the bicinchoninic acid method (Pierce, Rockford, IL, U.S.A.). Equal amounts of proteins (10–20 μg) were separated by SDS-PAGE (7.5%) and transferred to a nitrocellulose membrane (Pall Corporation, Ann Arbor, MI, U.S.A.). After blocking with 3% bovine serum albumin or 0.5% skim milk, membranes were incubated with primary antibody (1:200–1000 dilution) at 4°C overnight, and membrane-bound antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution, 1 hr) and the ECL-plus system (Amersham Biosciences, Buckinghamshire, UK). Equal loading of protein was confirmed by measuring total actin expression. The resulting autoradiograms were analyzed using CS Analyzer 3.0 software (ATTO, Tokyo, Japan).

Immunofluorescence staining: Immunofluorescence staining was performed as described previously [4]. HUVECs were fixed with 4% paraformaldehyde (pH 7.4) for 10 min at 4°C and permeabilized with 0.1% Triton X-100 for 15 min at room temperature. After blocking with 5% normal goat serum for 1 hr at room temperature, HUVECs were incubated with NF-κB p65 antibody (1:500 dilution, 2 hr) at room temperature followed by fluorescent-conjugated secondary antibody (Alexa Flour 488; 1:500 dilution, 1 hr, Invitrogen). Cells were nuclear stained with DAPI (1 μg/m*l*, 10 min, Dojindo). Images were obtained using fluorescence microscope (BX-51, Olympus) equipped with cooled CCD camera (MicroPulisher 5.0 RTV, Roper Japan, Tokyo, Japan).

RT-PCR: Total RNA was extracted from HUVECs by using QuickGene SP kit (Fuji Film, Tokyo, Japan) according to the instructions of the manufacturer. The first strand of cDNA was synthesized using random hexamers and Superscript II RT at 65°C for 5 min, 25°C for 2 min, 25°C for 10 min, 42°C for 50 min, and 70°C for 15 min. PCR amplification was performed using HotStarTaq Master Mix kit (Qiagen, Valencia, CA, U.S.A.). We used the following oligonucleotide primers pairs: for monocyte chemotactic protein-1 (MCP-1), sense 5'-AGTCTCTGCCGCCCTTCTGTG-3' and antisense 5'-TCTTCGGAGTTTGGGTTTGCTTGTC-3'; for tissue factor (TF), sense 5'-CGGCGCTTCAGGCACTACAAATA-3' and antisense 5'-TTCTCCTGGCCCATACACTCTAC-3'; for plasmogen activator inhibitor-1 (PAI-1), sense 5'-GTGGTTTTCTCACCCTATGG-3' and antisense 5'-GCAATGAACATGCTGAGGGT-3'; for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense 5'-GCTGATGCCCCCATGTTTG-3' and antisense 5'-GGGTGGTGGACCTCATGGC-3'. After initial activation at 95°C for 15 min, 28–33 cycles of amplifications at 94°C for 0.5 min, 43–52°C for 0.5 min, and 72°C for 1 min followed by final extension at 72°C for 10 min were done with a thermal cycler (PC707, ASTEC, Fukuoka, Japan). The expected size of MCP-1, TF, PAI-1 and GAPDH is 290, 648, 621, and 611 base pairs (bp), respectively. PCR products were electrophoresed on 2% agarose gel containing 0.1% ethidium bromide. Detectable fluorescent bands were visualized by an UV transilluminator and analyzed using CS Analyzer 3.0 software (ATTO).

Statistical analysis: Data are shown as mean ± SEM. Statistical evaluations were performed using one-way analysis of variance followed by Tukey's test. Values of $P<0.05$ were considered statistically significant.

RESULTS

Effects of vaspin on the morphological changes of HUVECs: We first examined morphological changes after treatment of HUVECs with vaspin. Treatment for 24 hr with vaspin (10 and 100 *n*g/m*l*) had no effects on HUVECs morphology (Fig. 1A). Since serum vaspin concentrations in normal individuals and patients with type 2 diabetes [36], carotid stenosis [2], chronic hemodialysis [25], or polycystic ovary syndrome [29] are less than 10 *n*g/m*l*, we chose the concentrations. Figure 1 B showed that in cells treated with TNF-α (10 *n*g/m*l*) for 24 hr, the cell density decreased and spindle typed cells were often observed. Pretreatment with vaspin (100 *n*g/m*l*) for 30 min did not inhibit TNF-α-induced morphological damage in HUVECs.

Effects of vaspin on TNF-α-induced activation of inflammatory signal transduction: Although we did not detect the influence of vaspin on the morphological changes in HUVECs, we hypothesized that vaspin could affect the cell signaling pathways resulting from TNF-α treatment. Activation of several kinases by Western blotting using phosphorylation-specific antibodies was examined. Treatment of HUVECs with TNF-α (10 *n*g/m*l*, 20 min) markedly induced activation of Akt, JNK and p38 (Fig. 2A). The fold increases of phoshorylation of Akt, JNK and p38 were 1.8 ± 0.2 (n=12), 7.3 ± 1.0 (n=4), 3.8 ± 0.5 (n=4), respectively, relative to control ($P<0.01$ vs. control). To explore whether activation of Akt, JNK and p38 is affected by vaspin, HUVECs were pretreated with vaspin (100 *ng/ml*, 30 min) before TNF-α stimulation (10 *n*g/m*l*, 20 min). However, pretreatment with vaspin did not suppress the phosphorylation of Akt, JNK or p38 induced by TNF-α, although a non-

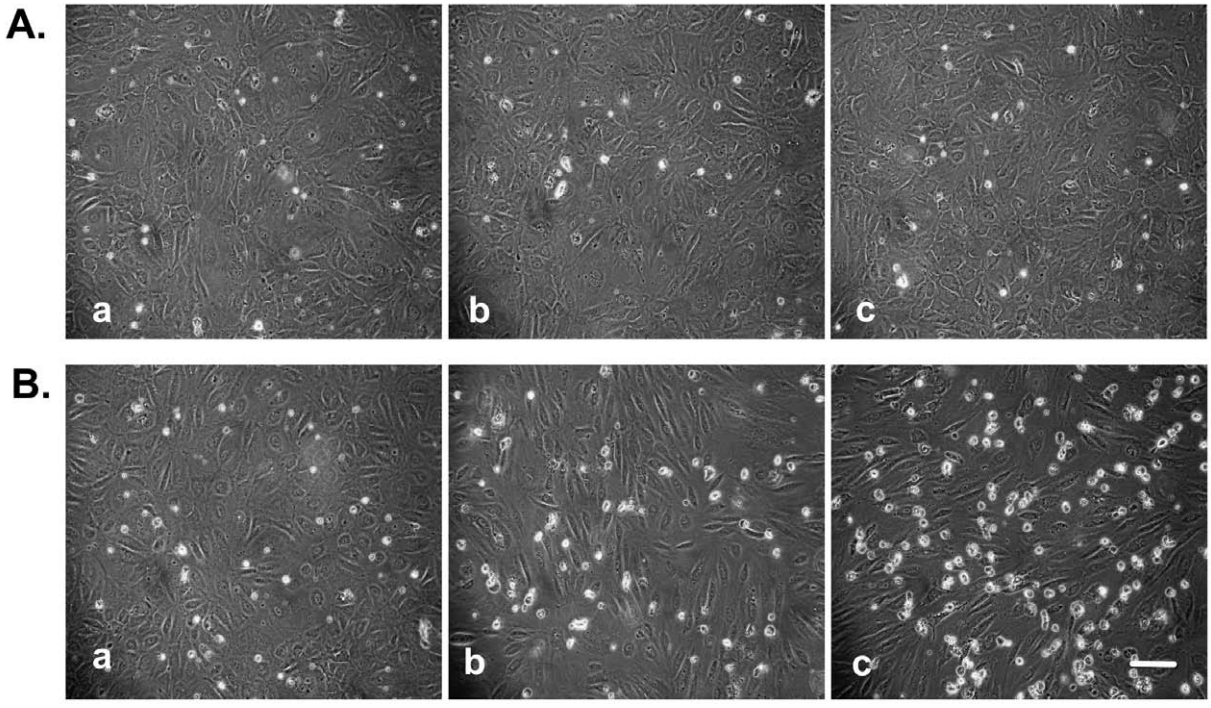

Fig. 1. Representative photomicrographs of HUVECs treated for 24 hr with (A) vaspin (a: 0; b: 10; c: 100 *n*g/m*l*) and of HUVECs treated for 24 hr with (B) TNF-α (a: 0; b, c: 10 *n*g/m*l*) in the presence of vaspin (100 *n*g/m*l*, pretreatment 30 min, c) were shown. Bar=50 μm.

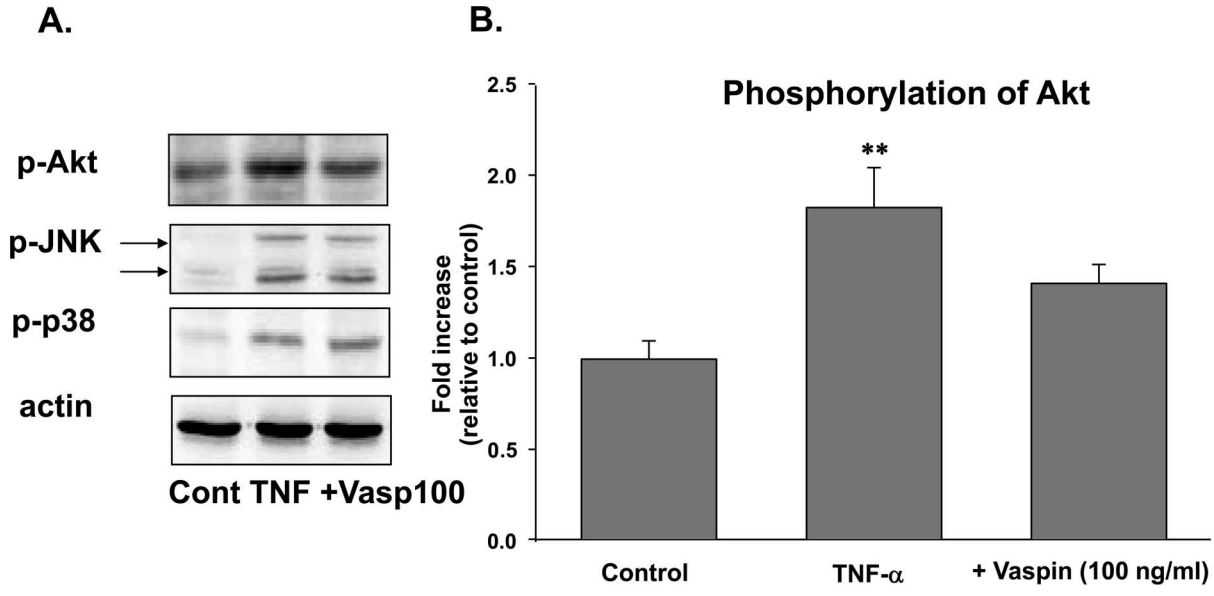

Fig. 2. (A) Effects of vaspin on TNF-α-induced phosphorylation of Akt, JNK, and p38 in HUVECs. (B) Phosphorylation of Akt is shown as fold-increase relative to control. After HUVECs at approximately 90% confluence were treated for 20 min with 10 *n*g/m*l* TNF-α in the absence or presence of vaspin (100 *n*g/m*l*; pretreatment for 30 min), total cell lysates were harvested. Activation of Akt, JNK, and p38 were determined by Western blotting using phospho-specific antibodies (Akt, n=12; JNK, and p38, n=4–8). Equal protein loading was confirmed by total actin antibody. Cont: Control; TNF: TNF-α (10 *n*g/m*l*); +Vasp100: TNF-α+vaspin (100 *n*g/m*l*). **: $P<0.01$ vs. Control.

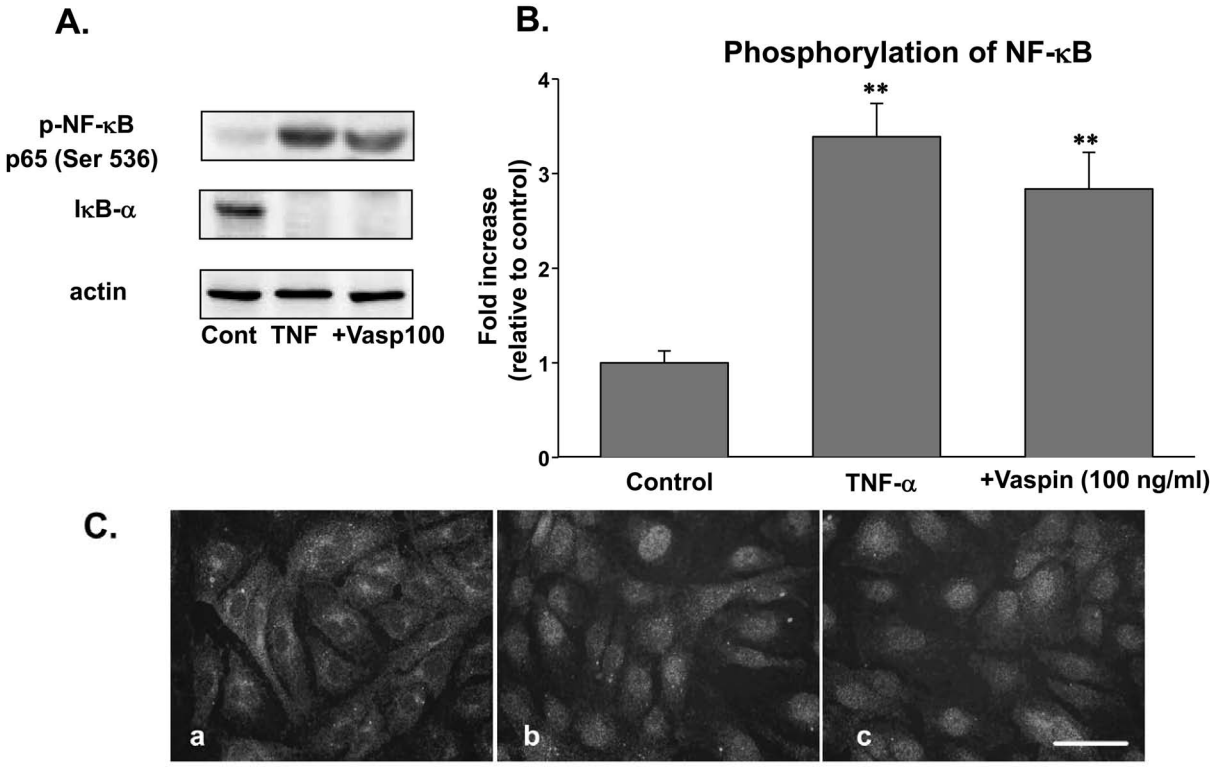

Fig. 3. Effects of vaspin on NF-κB pathway. (A) After HUVECs were treated for 20 min with 10 *n*g/m*l* TNF-α in the presence or absence of vaspin (100 *n*g/m*l*, pretreatment 30 min), total cell lysates were harvested. Activation of NF-κB was determined by measuring phosphorylation of NF-κB p65 at Ser536 as well as degradation of IκB-α protein. Equal protein loading was confirmed using total actin antibody (n=12). (B) Phosphorylation of NF-κB is shown as fold-increase relative to control. (C) Nuclear translocation of NF-κB p65 was examined by immunofluorescence staining after HUVECs were treated for 20 min with TNF-α (a: 0; b, c: 10 *n*g/m*l*) in the presence of vaspin (100 *n*g/m*l*, pretreatment 30 min, c) (n=4). Bar=50 μm. Cont: Control; TNF: TNF-α (10 *n*g/m*l*); +Vasp100: TNF-α+vaspin (100 *n*g/m*l*). **: P<0.01 vs. Control.

significant trend towards inhibition of the phosophorylation of Akt (1.4 ± 0.1 fold relative to control, n=12, Fig. 2B) was shown. In addition, vaspin (10 and 100 *n*g/m*l*, 20 min) did not affect the phosphorylation of Akt, JNK, or p38 in HUVECs without TNF-α stimulation (n=4, data not shown).

We next examined the activation of NF-κB pathway. Phosphorylation of NF-κB p65 at Ser536 was markedly induced by TNF-α treatment (10 *n*g/m*l*, 20 min, 3.4 ± 0.4 fold relative to control, P<0.01 vs. control, n=12, Fig. 3A and B). Vaspin pretreatment (100 *n*g/m*l*, 30 min) before TNF-α stimulation suppressed the phosphorylation of NF-κB by TNF-α to a certain extent but not significantly (2.8 ± 0.4 fold relative to control, n=12, Fig. 3A and B). Accordingly, degradation of IκB-α protein induced by TNF-α was not inhibited by vaspin pretreatment, as shown in Fig. 3A. We also observed that nuclear translocation of NF-κB p65 induced by TNF-α was not suppressed by vaspin pretreatment (100 *n*g/m*l*, 30 min, n=4, Fig. 3C).

Effects of vaspin on Akt- and NF-κB-related downstream inflammatory molecules: To verify whether the effects of vaspin treatment on phosphorylation of Akt and NF-κB impacts Akt- and NF-κB-related protein or gene expression, some inflammation- or endothelial cell dysfunction-associated molecules were studied by Western blotting or semi-quantitative RT-PCR analysis. As shown in Fig. 4A and B, stimulation with TNF-α (10 *n*g/m*l*, 24 hr) markedly enhanced VCAM-1, ICAM-1, e-selectin and COX-2 protein expression (3.7 ± 0.4 fold, 10.4 ± 2.4 fold, 3.2 ± 0.5 fold, 17.4 ± 2.4 fold, respectively, relative to control, n=4–8, P<0.01 vs. control) as well as MCP-1, TF, and PAI-1 mRNA expression (5.0 ± 0.3 fold, 3.6 ± 0.2 fold, 1.5 ± 0.2 fold, respectively, relative to control, n=6, P<0.01 vs. control) in HUVECs. Pre-incubation of HUVECs with vaspin (100 *n*g/m*l*, 30 min), however, had no effects on the induction of these molecules. Treatment of HUVECs with TNF-α (10 *n*g/m*l*, 24 hr) significantly decreased the eNOS expression (0.48 ± 0.1 fold, relative to control, n=8, P<0.01 vs. control). The inhibitory effect of TNF-α on eNOS was not reversed by vaspin pretreatment (100 *n*g/m*l*, 30 min).

Moreover, we investigated the effects of 100 *n*g/m*l* vaspin on HUVECs stimulated with lower concentration of TNF-α (2 *n*g/m*l*, for 20 min or 24 hr) as well as the effects of higher concentration (1 μg/m*l*) of vaspin on HUVECs

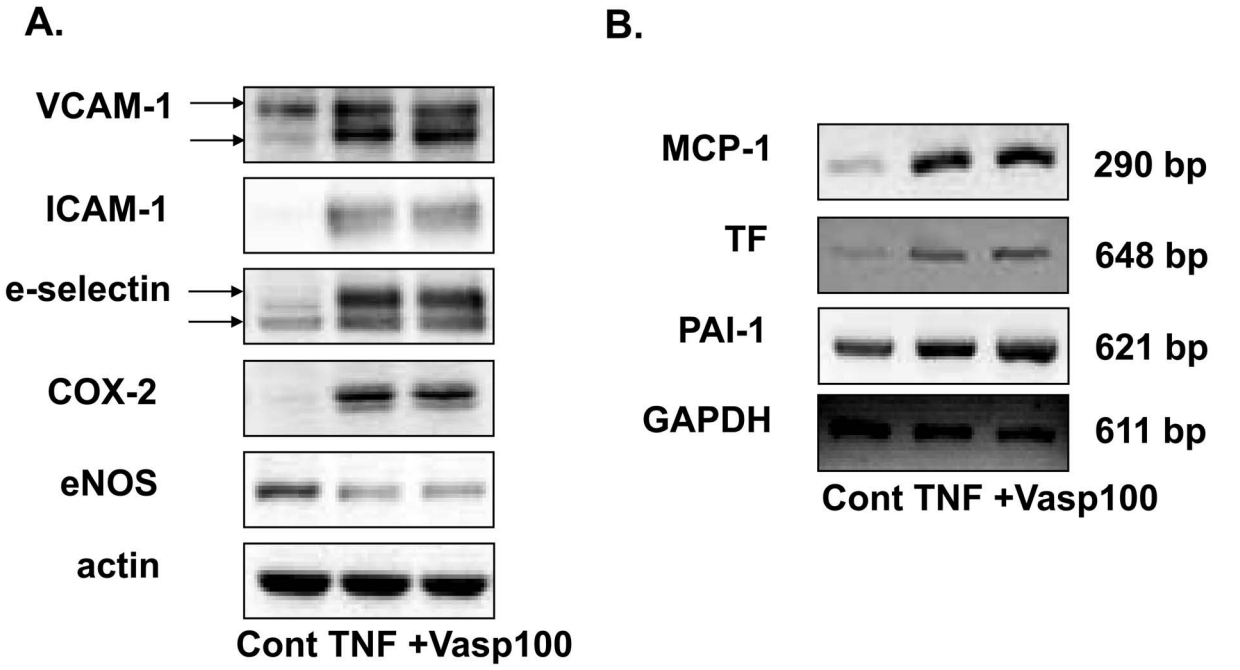

Fig. 4. Effects of vaspin on TNF-α-induced downstream molecules for Akt and NF-κB in HUVECs. After HUVECs were treated for 24 hr with 10 *n*g/m*l* TNF-α in the absence or presence of vaspin (100 *n*g/m*l*, pretreatment 30 min), total cell lysates were harvested. (A) Expressions of VCAM-1, ICAM-1, e-selectin, COX-2, and eNOS were determined by Western blotting. Equal protein loading was confirmed using total actin antibody (n=4–8). (B) Effects of vaspin on TNF-α-induced expression of monocyte chemotactic protein-1 (MCP-1, 290 bp), tissue factor (TF, 648 bp), and plasmogen activator inhibitor-1 (PAI-1, 621 bp) mRNA. After HUVECs were treated for 24 hr with 10 *n*g/m*l* TNF-α in the absence or presence of vaspin (100 *n*g/m*l*, pretreatment 30 min), total RNA was harvested. Expressions of MCP-1, TF, and PAI-1 mRNA were determined by RT-PCR analysis using the gene specific primers to human MCP-1, TF, and PAI-1. Equal loading of template cDNA was confirmed by ensuring the GAPDH (611 bp) expression (n=6). Cont: Control; TNF: TNF-α (10 *n*g/m*l*); +Vasp100: TNF-α+Vaspin (100 *n*g/m*l*).

stimulated with TNF-α (10 *n*g/m*l*, for 20 min or 24 hr). However, we also found that vaspin has no effects on these stimuli (data not shown).

DISCUSSION

In the present study, we investigated the effects of vaspin on basal and TNF-α-stimulated HUVECs. We found that vaspin treatment (100 *n*g/m*l*, 24 hr) had no effects on unstimulated HUVECs. When endothelial cells are exposed to inflammatory cytokines such as TNF-α, endothelial function can be altered or impaired [38]. Similarly, we also observed that TNF-α treatment (10 *n*g/m*l*, 24 hr) decreased the cell density and spindle typed cells appeared. However, pretreatment with vaspin (100 *n*g/m*l*, 30 min) did not prevent the morphological changes of HUVECs induced by TNF-α.

Adipokines actively participate in inflammatory and metabolic responses [31]. Hida *et al.* [11] reported that after obese ICR mice fed with high fat and sucrose chow were treated for 2 weeks with recombinant human vaspin (i. p.), expressions of TNF-α, leptin, and resistin were suppressed in mesenteric and subdermal white adipose tissues. Thus, we hypothesized that vaspin as a novel adipokine could also affect the TNF-α-activated inflammatory mediators in endothelial cells. To attest this assumption, we examined the effects of vaspin on some kinases and inflammation-related molecules induced by TNF-α. Interestingly, we found that pretreatment of HUVECs with vaspin (100 *n*g/m*l*, 30 min) slightly inhibited the phosphorylation of Akt induced by TNF-α (from 1.8 ± 0.2 fold to 1.4 ± 0.1 fold, relative to control). This result suggests that vaspin could directly interact with Akt or elements upstream of Akt, such as phosphotidylinositol 3-kinase (PI3K) and phosphatase and tensin homology deleted on chromosome ten (PTEN) [6]. It is well known in cancer cells that activation of Akt is enhanced as a survival/proliferative signal and the inhibition of Akt activity induces apoptosis [7, 21]. Thus, it is possible that vaspin may be a potential tumor suppressor like maspin, another member of serpin superfamily [23].

NF-κB plays a key role in inflammatory responses and can be activated by TNF-α through both the classical IKK/IκB-α pathway and PI3K/Akt signaling in endothelial cells [13, 15, 38]. In our study, TNF-α-induced NF-κB activation was mainly dependent on IKK cascade, which was verified by the nuclear translocation of NF-κB p65 and IκB-α degradation. However, vaspin did not inhibit the nuclear translocation of NF-κB p65 and IκB-α degradation by TNF-

α. On the other hand, phosphorylation of NF-κB was suppressed by vaspin to a certain extent (from 3.4 ± 0.4 fold to 2.8 ± 0.4 fold, relative to control), although not significantly. According to the signaling pathway described above, it is possible that the decrease of NF-κB activation by vaspin may be due to the inhibitory effects of vaspin on the phosphorylation of Akt.

Some inflammatory mediators including VCAM-1, ICAM-1 [37], e-selectin [12], COX-2 [16], MCP-1 [1], TF [15] and PAI-1 [17] are induced by the activation of JNK, p38, or NF-κB. In the present study, we also found that TNF-α induced the activation of JNK, p38, and NF-κB, the protein expression of VCAM-1, ICAM-1, e-selectin and COX-2, and the mRNA expression of MCP-1, TF and PAI-1. Vaspin pretreatment did not significantly inhibit the activation of JNK, p38, NF-κB, and the induction of these inflammatory molecules in TNF-α-stimulated HUVECs. Although these data are contradictory to the reports of Hida *et al.* [11] that *in vivo* vaspin treatment to mice suppressed TNF-α expression, it might be due to the different experimental settings (*in vitro* cultured cells vs. *in vivo* whole animals). Alternatively, it might be possible that vaspin could be effective to another inflammatory stimulus. However, it is not likely since we found that vaspin (100 *n*g/m*l*) had no effect on hydrogen peroxide (30–300 μM for 1–6 hr)-induced HUVECs inflammation (data not shown).

In the present study, the mechanism by which vaspin affects cultured cells *in vitro* is unknown. It was previously reported that the serpin-enzyme complex (SEC) recognizes the α1-antitrypsin (another serine protease inhibitor)-elastase complexes, then mediates internalization and intracellular catabolism of the macromolecular complex, and elicits neutrophil chemotactic activity [14]. Therefore, it is conceivable that similar to α1-antitrypsin, vaspin might interact with the SEC receptor or its own specific receptors to induce the cellular signaling.

In summary, we demonstrated that vaspin had no effects on normal HUVECs, did not inhibit inflammation of HUVECs induced by TNF-α, but slightly inhibited the phosphorylation of Akt induced by TNF-α. Further investigations of the pathophysiological roles of vaspin may be helpful to new pharmacotherapeutic approaches to obesity-associated cardiovascular diseases.

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