Visceral Adipose Tissue-derived Serine Proteinase Inhibitor Inhibits Apoptosis of Endothelial Cells as a Ligand for the Cell-Surface GRP78/Voltage-dependent Anion Channel Complex


**Rationale:** Visceral adipose tissue-derived serine proteinase inhibitor (vaspin) is an adipokine identified from visceral adipose tissues of genetically obese rats.

**Objective:** The role of vaspin in the diabetic vascular complications remains elusive, and we investigated the effects of vaspin on the vascular function under the diabetic milieu.

**Methods and Results:** Adenovirus carrying the full length of the vaspin gene (Vaspin-Ad) ameliorated intimal proliferation of balloon-injured carotid arteries in diabetic Wistar rats. The expression of Ccl2, Pdgfb, and Pdgfrb genes was significantly reduced by the treatment of Vaspin-Ad. In cuff-injured femoral arteries, the intimal proliferation was ameliorated in vaspin transgenic (Vaspin Tg) mice. The application of recombinant vaspin and Vaspin-Ad promoted the proliferation and inhibited the apoptosis of human aortic endothelial cells. Adenovirus expressing vaspin with calmodulin and streptavidin-binding peptides was applied to human aortic endothelial cells, subjected to tandem tag purification and liquid chromatography-tandem mass spectrometry, and we identified GRP78 (78-kDa glucose-regulated protein) as an interacting molecule. The complex formation of vaspin, GRP78, and voltage-dependent anion channel on the plasma membrane was confirmed by the immunoprecipitation studies using aortas of Vaspin Tg mice. The binding assay using 125I-vaspin in human aortic endothelial cells revealed high-affinity binding (dissociation constant = 0.565×10⁻⁹ m) by the treatment of 5 μM thapsigargin, which recruited GRP78 from the endoplasmic reticulum to plasma membrane by inducing endoplasmic reticulum stress. In human aortic endothelial cells, vaspin induced phosphorylation of Akt and inhibited the kringle 5-induced Ca²⁺ influx and subsequent apoptosis.

**Conclusions:** Vaspin is a novel ligand for the cell-surface GRP78/voltage-dependent anion channel complex in endothelial cells and promotes proliferation, inhibits apoptosis, and protects vascular injuries in diabetes mellitus. ([Circ Res. 2013;112:771-780.])

**Key Words:** apoptosis ■ atherosclerosis ■ diabetes mellitus ■ endothelium

Visceral adipose tissue-derived serine proteinase inhibitor (vaspin), belonging to serpin clade A (SerpinA12), was cloned from visceral adipose tissues of genetically obese rats, and it is a compensatory adipokine to improve insulin sensitivity with anti-inflammatory properties demonstrated by recombinant human vaspin protein (Vaspin) administration into diet-induced obesity mice.1,2 In later human studies, elevated serum vaspin concentrations in obesity, type 2 diabetes mellitus,4,5 and polycystic ovary syndrome6 were reported, and it correlates with insulin resistance and C-reactive protein levels.4,7,8 In addition, low vaspin serum concentrations correlate with recently experienced ischemic events in patients with...
Nonstandard Abbreviations and Acronyms

<table>
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<th>Abbreviation</th>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>GRP78</td>
<td>78-kDa glucose-regulated protein</td>
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<tr>
<td>HAEC</td>
<td>Human aortic endothelial cells</td>
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<tr>
<td>HASMC</td>
<td>Human aortic smooth muscle cells</td>
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<tr>
<td>MCP-1</td>
<td>Macrophage chemoattractant protein-1</td>
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<td>MTJ-1</td>
<td>Murine tumor cell DNAJ-like protein-1</td>
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<td>PDGF</td>
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<td>shRNA</td>
<td>Short hairpin RNA</td>
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<td>Vaspin</td>
<td>Visceral adipose tissue-derived serine protease inhibitor</td>
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carotid stenosis. Decreased vaspin serum levels were also observed in asymptomatic patients with coronary artery disease, and low vaspin concentrations seemed to correlate with coronary artery disease severity.

GRP78 is an endoplasmic reticulum (ER) chaperone that binds to hydrophobic patches of nascent polypeptides in ER and serves as a switch for the induction of unfolded protein response. GRP78 directly binds to inositol requiring enzyme 1α, activating transcription factor-6, and protein kinase R-like eukaryotic initiation factor 2α kinase under normal condition. Under ER stress, the available pool of GRP78 is occupied by preventing the aggregation of unfolded proteins, and GRP78 releases inositol requiring enzyme 1α, activating transcription factor-6, and protein kinase R-like eukaryotic initiation factor 2α kinase, which are transducers of the unfolded protein response signals. Beyond the ER retention, GRP78 is also associated with various anchor membrane proteins on the cell surface and functions as a receptor for various ligands and a signaling hub for either in cell survival or cell death.

Recently, we demonstrated that vaspin ameliorates ER stress and insulin resistance under obese states by acting as a ligand for cell-surface GRP78/MTJ-1 (murine tumor cell DNAJ-like protein-1) complex in the liver. Here, we show vaspin exerts antiapoptotic potential to the endothelial cells and it protects from the vascular injuries in a balloon-injured model in Wistar rats with streptozotocin-induced diabetes mellitus. Furthermore, we demonstrate that vaspin is a ligand for cell-surface-associated GRP78/voltage-dependent anion channel (VDAC) complex and give a new functional insight and molecular basis of the direct crosstalk between an adipokine and ER stress responses of endothelial cells.

Methods

Mice and Rats

Male Wistar rats at 14 weeks of age (Charles River) were treated with a single injection of 100 mg/kg of streptozotocin (STZ) (Sigma) in citrate buffer at pH 4.6. Three days after STZ injection, hyperglycemia (>15 mmol/L) was confirmed and daily administration of neutral protamine Hagedorn insulin at a dosage of 5 units per kilogram of weight was administrated daily and continued up to 10 weeks of age. We performed cuff-injury experiments of femoral arteries as reported previously, and intimal proliferation was investigated. All of the animal procedures were in accordance with the Guide for Care and Use of Laboratory Animals at Okayama University Department of Animal Resources Advanced Science Research Center.

Preparation of Adenovirus Expression Vector and Recombinant Human Vaspin

Adenoviruses carrying the full length of mouse vaspin (Vasin-Ad), vaspin tagged with calmodulin, and streptavidin-binding peptides (pCTAP-Vasin-Ad) (InterPlay Mammalian TAP system, Stratagene) and β-galactosidase (LacZ) were prepared by Adenovirus Expression Kit (Takara), as described. β-Galactosidase activity was demonstrated with β-Gal Staining Set (Roche). Human vaspin was expressed in Escherichia coli using a pET expression system (pET16b, Novagen), and (His)6-tagged vaspin was purified by an nickel-nitrilotriacetic acid affinity column. (His)6-tag was removed by Factor-Xa digestion and further purified by cation-exchange column chromatography. Endotoxin was removed by Acticlean Etox column (Stergene Bioseparations). Final endotoxin concentration of purified vaspin was <0.1 EU/mg of protein, as measured by using the limulus amoebocyte lysate assay (Seikagaku Corp).

Cell Culture

Human aortic endothelial cells (HAECs) and human aortic smooth muscle cells (HASMCs) isolated from thoracic aorta were cultured using EGM-2 BulletKit and SmGM2 BulletKit (Takara). HAECs and HASMCs, 1x10⁴ cells per well in 24-well culture plates or 5x10² cells per well in 96-well culture plates, were cultured for 24 hours and transfected with adenovirus vectors or treated with Vaspin (10 ng/mL) and further cultured for 9 days. Cell proliferation ELISA using 5-bromo-2′-deoxyuridine (Roche) at 24 hours, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based colorimetric assay for the cell proliferation and viability (Roche) at day 3, and apoptosis assay (TiterTACS, Trevigen) at day 3 were performed. Final cell counts at day 9 were measured with a Coulter counter (model Z1, Beckman Coulter).

HAECs were treated with various concentrations of Vaspin (1–100 ng/mL) and Western blot analysis was performed using Phospho-Akt (Ser473) and Akt (Cell Signaling Technology) antibodies. The Vaspin concentrations from 1 to 100 ng/mL were used because human serum concentrations ranged from 0.1 to 100.0 ng/mL. Recombinant human kringles 5 and 8 were measured with a Coulter counter model Z1, Beckman Coulter.

For quantitative real-time polymerase chain reaction analysis, cDNA was synthesized from 2 μg of total RNA was analyzed in a Sequence Detector (model 7900; PE Applied Biosystems) with specific primers and SYBR Green PCR Master (Perkin Elmer Life Sciences). The relative abundance of mRNAs was standardized with 36B4 mRNA as the invariant control. The mRNA expression was determined by SYBR green, and statistical analysis of mRNA data was performed by the Student’s t test. Gene specific primers are indicated in Table S1, available in the online-only Data Supplement.

Poly(A⁺) RNA Analysis

For quantitative real-time polymerase chain reaction analysis, cDNA synthesized from 2 μg of total RNA was analyzed in a Sequence Detector (model 7900; PE Applied Biosystems) with specific primers and SYBR Green PCR Master (Perkin Elmer Life Sciences). The relative abundance of mRNAs was standardized with 36B4 mRNA as the invariant control. The mRNA expression was determined by SYBR green, and statistical analysis of mRNA data was performed by the Student’s t test. Gene specific primers are indicated in Table S1, available in the online-only Data Supplement.
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Western Blot Analysis
Aortas of Vaspin Tg and Vaspin−/− mice were removed and fractionated by discontinuous iodixanol gradients following protocol S36 of the manufacturer's application sheets (OptiPrep, Invitrogen). To investigate the vaspin-GRP78-VDAC complex in plasma membranes, cell surface proteins of HAECS were biotinylated and isolated by Pierce Cell Surface Protein Isolation Kit (Thermo Scientific). In addition, the protein lysates of total and subcellular fractions of aortas and HAECs were subjected to SDS-PAGE and transferred to polyvinylidine fluoride membranes. The membranes were treated with specific antibodies (1:200–1000 dilution), including GRP78 (C20), VDAC1 (20B12), vaspin (I-14; Santa Cruz), vaspin (Strategic Biosolutions), phospho-Akt (Ser473), Akt, macrophage chemoattractant protein-1 (MCP-1), platelet-derived growth factor receptor-β (PDGFR-β), GAPDH (Cell Signaling Technology), and platelet-derived growth factor (PDGF; Abcam) overnight at 4°C, followed by the treatment with secondary antibodies conjugated with horseradish peroxidase, and finally visualized with ECL Plus (GE Healthcare) and luminescent image analyzer (LAS-3000mini, Fujifilm). Immunoprecipitation was performed using a Universal Magnetic CoIP kit (Active Motif).

High-Performance Liquid Chromatography-Tandem Mass Spectrometry
Adenovirus vector expressing vaspin tagged with calmodulin and streptavidin-binding peptides (pCTAP-Vaspin-Ad) was introduced to HAECs. Soluble proteins were purified by CBP and SBP binding AB G H I CD EF Figure 1. Balloon injuries of carotid arteries treated with adenovirus vector expressing LacZ (LacZ) and visceral adipose tissue-derived serine protease inhibitor (vaspin; Vaspin-Ad) in streptozotocin-induced diabetic Wistar-Kyoto (WKY) rats. A through H, Immuno staining of macrophage chemotactant protein-1 (MCP-1), platelet-derived growth factor receptor-β (PDGFR-β), GAPDH (Cell Signaling Technology), and platelet-derived growth factor (PDGF; Abcam) overnight at 4°C, followed by the treatment with secondary antibodies conjugated with horseradish peroxidase, and finally visualized with ECL Plus (GE Healthcare) and luminescent image analyzer (LAS-3000mini, Fujifilm). Immunoprecipitation was performed using a Universal Magnetic CoIP kit (Active Motif).

Figure 2. Balloon injuries of carotid arteries treated with adenovirus vector expressing LacZ (LacZ) and visceral adipose tissue-derived serine protease inhibitor (vaspin; Vaspin-Ad) in streptozotocin-induced diabetic Wistar-Kyoto (WKY) rats. A through H, Immunostaining of macrophage chemotactant protein-1 (MCP-1; A and B), platelet-derived growth factor (PDGF)-B (C and D), platelet-derived growth factor receptor-β (PDGFR-β; E and F), and Ki-67 (G and H). I, Quantitative reverse-transcription polymerase chain reaction for MCP-1, PDGF-B, and PDGFR-β genes in carotid arteries. Bars is 50 μM in A through F, and 100 μM in G and H. All of the data are presented as mean±SEM. *P<0.05 vs LacZ.
resin (InterPlay Mammalian TAP System, Stratagene) and subjected to SDS-PAGE and Coomassie blue staining. Visible bands were excised and in-gel digested with trypsin and analyzed with liquid chromatography-tandem mass spectrometry.20

Pull-down Assay and 125I-Vaspin Binding Analysis
Cod ing sequences without the signal peptide of GRP78 19-654 were ligated to KpnI-XhoI and SalI-NorI sites of the pTNT vector (Promega). Coding sequence without signal peptide of vaspin was ligated to the BamHI-XhoI site of the pET-42a vector. Pull-down assay was performed using the MagneGST Pull-Down System (Promega). Vaspin was iodinated with specific activity of 7.8 μCi/μg (PerkinElmer) and used for a solid phase assay. Cell binding assay of cultured HAECS was performed using 125I-vaspin and subjected to Scatchard analysis in the presence of various concentrations of Vaspin and kringle 5.

Light Microscopic Examinations, Immunohistochemistry, and TUNEL Assay
Tissues were fixed in 10% formaldehyde and embedded in paraffin, and 4-μm-thick sections were prepared. The sections were stained with periodic acid-Schiff and Elastica van Gieson staining. The areas of intima and media were measured with image analysis software (LuminaVision). Four-micrometer-thick sections of formalin-fixed paraffin-embedded tissues were deparaffinized and rehydrated, and endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide and methanol. Sections were pretreated for 30 minutes in 10% rabbit serum. The tissues were then incubated with a biotinylated secondary antibody, ABC Elite Reagent (Vector Laboratories). After PBS wash, sections were incubated with a biotinylated secondary antibody, ABC Elite Reagent (Vector Laboratories). Terminal deoxynucleotidyl transferase dUTP nick end labeling assay was performed using the DeadEnd Colorimetric TUNEL system (Promega).

Statistical Analysis
Data are expressed as the mean±SEM and analyzed by the unpaired Student t test. *P<0.05 was regarded as statistically significant. The data were analyzed with PASW Statistics 18 (SPSS Inc, Chicago, IL).

Results
Vaspin Ameliorates Intimal Proliferation and Inhibits the Apoptosis of Endothelial Cells
We investigated the effects of vaspin on vascular functions in diabetic rats. The mouse vaspin (Vaspin-Ad) and recombinant human vaspin (Vaspin) under normal glucose (5.5 mM), high glucose (25 mM), and osmotic control (mannitol; 5.5 mM glucose + 19.5 mM mannitol). A through D, Cell count (A), 5-bromo-2’-deoxyuridine (BrdU assay; B), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (C), and apoptosis assay (D) in HAECs treated with LacZ and Vaspin-Ad. E and F, Cell count (E) and BrdU assay (F) in HAECs treated with presence of 10 ng/mL of Vaspin or absence of Vaspin, Vaspin (–), *P<0.05, **P<0.01 vs LacZ-treated HAECs. G and H, BrdU assay (G) and MTT assay (H) in HAECs treated with vaspin, α1-antitrypsin (α1AT), and plasminogen activator inhibitor-1 (PAI-1). All of the data are presented as mean±SEM. n=5 to 7.
demonstrated successful delivery of LacZ and vaspin genes (Figure 1C, 1D, 1G, and 1H). Intima/media ratio was significantly reduced in the Vaspin-Ad–treated group compared with the LacZ-Ad–treated group (Figure 1I). The treatment of Vaspin-Ad reduced the number of MCP-1, PDGF-B, PDGFR-β, and Ki-67–positive cells in the proliferated intimal area, and it also reduced mRNA levels of these genes (Figure 2). Apoptotic endothelial cells were occasionally observed in balloon-injured carotid arteries; however, these cells were seldomly observed in those treated with Vaspin-Ad (Online Figure I). Furthermore, we performed the loss and gain of function experiments in vivo using Vaspin Tg and Vaspin–/– mice with a cuff-injury model of femoral arteries. Although the apparent deterioration of intimal proliferation was not observed in Vaspin–/– mice compared with wild-type mice (data not shown), significant reduction of the intima/media ratio was observed in Vaspin Tg mice. Because the serum concentration of vaspin was 0.64±0.14 ng/mL in Vaspin Tg, 0.21±0.01 ng/mL in wild-type mice, and undetected in Vaspin–/– mice, certain serum concentrations of vaspin were required in the protection of the cuff-injured model of femoral arteries (Figure 3).

In HAECs, the application of Vaspin-Ad significantly increased cell proliferation assessed by cell count, 5-bromo-2′-deoxyuridine, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays and inhibited apoptosis under high-glucose conditions compared with LacZ-Ad. The addition of Vaspin (10 ng/mL) into culture media also significantly increased cell proliferation (Figure 4A through 4F). In contrast, the other serpin members, such as α1-antitrypsin and plasminogen activator inhibitor-1, significantly inhibited cell proliferation (Figure 4G and 4H). In human aortic smooth muscle cells (HASMCs), the addition of Vaspin into the culture medium did not alter heparin-binding epidermal growth factor-, epidermal growth factor-, or basic fibroblast growth factor (bFGF)-induced proliferation (Figure 5A through 5D). However, the treatment of Vaspin-Ad, as well as Vaspin, significantly inhibited the insulin- and PDGF-induced cell proliferation under a high-glucose condition (Figure 5E through 5H). High glucose upregulated the expression of MCP-1, PDGF-B, and PDGFR-β, whereas the treatment of Vaspin reversed protein levels of MCP-1 in HAECs (Figure 6A). In HASMCs, the upregulation of MCP-1, PDGF-B, and PDGFR-β was not reproduced, and the treatment of Vaspin mainly reduced the expression of PDGF-B (Figure 6B).

**Vaspin Interacts With 78-kDa Glucose-Regulated Protein**

We further screened the interacting molecules of vaspin by tandem affinity tag purification. Adenovirus vector expressing

Figure 5. Human aortic smooth muscle cells (HASMCs) treated with adenovirus vector expressing LacZ (LacZ), visceral adipose tissue-derived serine protease inhibitor (vaspin; Vaspin-Ad) and recombinant human vaspin (Vaspin) under normal glucose (5.5 mM), high glucose (25 mM), and osmotic control (mannitol; 5.5 mM glucose + 19.5 mM mannitol). A and B, Cell count (A) and 5-bromo-2′-deoxyuridine (BrdU) assay (B) are not altered by the 10 ng/mL of Vaspin compared with negative control, Vaspin (–). C and D, Heparin-binding epidermal growth factor (HBEFG)-, epidermal growth factor (EGF)-, and basic fibroblast growth factor (bFGF)-induced proliferation are not altered by the treatment of 10 ng/mL of Vaspin. E through H, Insulin and platelet-derived growth factor (PDGF)-induced proliferation of HASMCs is inhibited by the treatment of Vaspin-Ad (E and F) or 10 ng/mL of Vaspin (G and H). All of the data are presented as mean±SEM. n=5–7. *P<0.05, **P<0.01 vs LacZ in E and F. *P<0.05. **P<0.01 vs Vaspin (–) in G and H.
Vaspin Is a Ligand for Plasma Membrane Associated GRP78

GRP78 is known to associate with VDAC on plasma membrane of endothelial cells, and kringle 5 is a ligand that increases intracellular Ca$^{2+}$ concentration and induces apoptosis. It has been reported that the recruitment of GRP78 from ER to cell surface is facilitated by the addition of thapsigargin into culture medium. Binding assay using $^{125}$I-vaspin and HAECs demonstrated the high-affinity binding of $^{125}$I-vaspin under the treatment of thapsigargin compared with no treated control and tunicamycin-treated HAECs (Figure 7D through 7F), suggesting that thapsigargin is an efficient inducer for the recruitment of GRP78 from ER to plasma membrane. The amount of GRP78 in total cell lysates increased by the treatment of high-glucose condition; however, its expression in plasma membrane fractions was unaltered. The treatments with thapsigargin increased the protein expression of GRP78 both in total lysates and plasma membrane fractions (Figure 7G). The pull-down assay also demonstrated the inhibition of the binding between glutathione S-transferase-Vaspin and GRP78 by kringle 5 (Figure 7H). The binding of $^{125}$I-vaspin to thapsigargin-treated HAECs was inhibited by the excess of cold vaspin, as well as kringle 5, in a dose-dependent manner (Figure 7I). The addition of Vaspin in the culture also increased the phosphorylation of Akt in HAECs in a dose-dependent manner (Figure 8A). The knockdown experiments using shRNA-GRP78 and shRNA-VDAC inhibited dose-dependent phosphorylation of Akt in HAECs by Vaspin treatments (Online Figure IVA). Vaspin-induced proliferation of HAECs was also suppressed by the treatment of shRNA-GRP78 and shRNA-VDAC (Online Figure IVB). The amelioration of kringle 5-induced apoptosis by Vaspin was also dose dependent (Figure 8B). Kringle 5 evoked the elevation of intracellular Ca$^{2+}$ concentration in HAECs, and the addition of Vaspin inhibited the rise in intracellular Ca$^{2+}$ concentration in a dose-dependent manner (Figure 8C). In vivo experiments, the expression of kringle 5 was observed in endothelial cells and smooth muscle cells in balloon-injured models of carotid arteries, and it was reduced by the treatment of Vaspin-Ad (Online Figure VA through VD). pAkt-positive cells were mainly endothelial cells, and they increased by the treatment of Vaspin-Ad (Online Figure VE and VF).

Discussion

In the current investigation, we clearly demonstrated that vaspin is a new ligand for the GRP78/VDAC complex in HAECs. GRP78 is known to associate with VDAC on plasma membrane of endothelial cells, and kringle 5 is a ligand, which increases intracellular Ca$^{2+}$ concentration and induces apoptosis. It has been reported that the recruitment of GRP78 from ER to cell surface is facilitated by the addition of thapsigargin into culture media. Although tunicamycin is the bona fide...
ER stress inducer, tunicamycin did not promote the binding of vaspin to plasma membrane. Because tunicamycin inhibits N-linked glycosylation and thapsigargin leads to ER Ca\(^{2+}\) depletion because of inhibition of the sarco/ER Ca\(^{2+}\)-ATPase, the alteration of Ca\(^{2+}\) metabolism of ER may be important for the translocation of GRP78 in addition to the process of ER

**Figure 7.** Colocalization of visceral adipose tissue-derived serine protease inhibitor (vaspin) with GRP78 and voltage-dependent anion channel (VDAC) in plasma membrane and binding assays. A, Adenovirus vector expressing vaspin C-terminal tagged with calmodulin and streptavidin-binding peptides (pCTAP-Vaspin-Ad) was applied to human aortic endothelial cells (HAECs), and purified products are analyzed by SDS-PAGE. In-gel digestion with trypsin and analyzed by liquid chromatography-tandem mass spectrometry revealed that band 1 is GRP78 and band 2 is vaspin. B, HAECs treated with 10 ng/mL of Vaspin under 5 \(\mu\)M thapsigargin. Cell surface proteins were biotinylated and isolated by Pierce Cell Surface Protein Isolation Kit and subjected to Western blot analyses. C, Aorta from mice was fractionated by discontinuous iodixanol gradients, and plasma membrane fractions were immunoprecipitated with anti-vaspin antibody and immunoblotted with anti-GRP78 (C20) and anti-VDAC1 antibodies. D through F, High-affinity binding of \(^{125}\)I-vaspin is observed in cultured human aortic endothelial cells (HAECs) by the treatment with thapsigargin and not by tunicamycin. Thapsigargin is known to increase cell-surface GRP78 in HAECs. The binding is competed by cold Vaspin. G, Western blot analyses of GRP78 in total cell lysates and plasma membrane fractions in HAECs treated with high glucose or thapsigargin. H, The specific binding glutathione S-transferase (GST)-Vaspin fusion protein and in vitro translated GRP78 (pTNT-GRP78) are demonstrated by pull-down assay. The specific binding is competed by kringle 5 (K5), the known ligand for the GRP78/VDAC complex in HAECs. All of the values are presented as mean±SEM. n=3 to 5. *P<0.05, **P<0.01 vs K5 (–).
and promote the survival of vascular endothelial cells. In HAECs, Vaspin induced upregulation of pAkt, antagonized with kringle 5, and inhibited the kringle 5-induced Ca^{2+} influx and apoptosis. Because GRP78 is recruited under the ER stress, vaspin modulated the ER stress-induced dysregulation of endothelial cells, such as cell death and proinflammatory responses. Actually, vaspin inhibited the kringle 5-induced Ca^{2+} influx and apoptosis in cultured HAECs, and overexpression of vaspin ameliorated the intimal proliferation in diabetic Wistar rats and C57BL/6Jc1 mice.

It is quite interesting to note that we can modulate the ER stress by approaching from outside of the cells by interacting with GRP78, which is recruited from ER to the cell surface. However, GRP78 does not possess the hydrophobic membrane domain and should be associated with various membrane-anchored proteins. Thus, the combination of GRP78, membrane-anchored proteins, and ligands is rather complex, and they demonstrated diverse subsequent biological events. In many tumor cells, α2M binds to cell-surface GRP78/MTJ-1, activates phosphatidylinositol 3-kinase/Akt signal to suppress apoptotic pathways, concomitantly upregulates nuclear factor-κB, and induces unfolded protein response so that cell proliferation occurs. In addition to α2M, teratocarcinoma-derived growth factor-1 (Cripto) and GRP78 form a complex at the cell surface and enhance the cell growth by inhibiting transforming growth factor-β signaling events. Truncated cadherin and GRP78 exert the activation of Akt and promote the survival of vascular endothelial cells. In contrast, the association of GRP78 with plasminogen kringle 5 domain or extracellular Par-4 promotes apoptosis in vascular endothelial cells and tumor cells. VDAC is the major permeability pathway in the mitochondria outer membrane, and its closure, which induces apoptosis, also favors Ca^{2+} flux into mitochondria. VDAC is also associated with GRP78 and localized on plasma membranes, and kringle 5 is a ligand for GRP78/VDAC inducing a small rise in intracellular Ca^{2+} and apoptosis. In vascular endothelial cells, vaspin interacts with GRP78/VDAC, antagonizes with kringle 5, inhibits Ca^{2+} influx, and induces prosurvival signals, such as inhibition of apoptosis and enhanced cell proliferation. Thus, vaspin modulates the ER stress-induced responses through an outside-in signal by interacting with cell surface GRP78, and it has beneficial effects by ameliorating the vascular injuries in diabetes mellitus. We demonstrated that the signals downstream of Vaspin-GRP78/VDAC pathways induce the phosphorylation of Akt. Recent publication supported our findings in which vaspin inhibited free fatty acid-induced apoptosis by activating the Akt pathway, and the protective effects of vaspin were cancelled by a phosphatidylinositol 3-kinase inhibitor. Taken together, vaspin exerts antiapoptotic actions in vascular endothelial cells via the Vaspin-GRP78/VDAC pathway (Online Figure V).

In addition to HAECs, vaspin demonstrated inhibitory effects on proliferation and inflammatory responses in HASMCs. In our investigations, vaspin suppresses the proinflammatory responses such as MCP-1, PDGFRβ, and PDGF-B in injured vascular cells. Recent publications also supported our observations; vaspin inhibited PDG-F–induced migration of vascular smooth muscle cells and it also prevented the expression of tumor necrosis factor-α–induced intercellular adhesion molecule-1 by inhibiting reactive oxygen species–induced nuclear factor-κB in cultured rat vascular smooth muscle cells. The exact mechanism of the opposite role of vaspin on proliferation of HAECs and HASMCs is not well characterized in the current investigation; we can speculate on the combination of the cell surface complex of GRP78 and corresponding anchor proteins.

The current investigation would open a new avenue for the development of therapeutic modalities to treat the vascular
complications in patients with diabetes mellitus. Anti-GRP78 antibodies and small molecules interacting with GRP78, which mimic the action of vaspin, may be beneficial for the treatment of diabetic vascular diseases. We need to further evaluate the antiapoptotic activity of vaspin on various tissues and cell types and identify the unknown partners of GRP78 on plasma membranes, because vaspin may exert antiapoptotic and anti-inflammatory effects by acting on unknown combinations of the GRP78 complex and modulating ER stress responses. In addition, we should further elucidate whether the beneficial effects of vaspin on vascular injuries are specific in diabetic vascular diseases or also present in other causes, because vaspin revealed proliferative actions on mannitol treated in cultured HAECs. The poor prognosis of the majority of tumors correlates with the expression of cell surface GRP78 and the presence of autoantibodies, which mediates prosurvival signals and responses to cellular stress. Thus, blocking anti-GRP78 antibodies, small molecule inhibitors, anticancer agent conjugated with vehicle targeted to GRP78, or GRP78 promoter-driven expression of suicide genes may be expected in the treatment of cancers. Vaspin indeed has a beneficial role in ameliorating vascular dysfunction; future studies are required to address the concern that vaspin or its mimetics may promote the tumor growth by acting on the cell surface GRP78 on tumors cells.

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Disclosures

None.

References


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**Novelty and Significance**

**What Is Known?**

- Vaspin is an adipokine with insulin-sensitizing action and anti-inflammatory properties. Vaspin ameliorates ER stress and insulin resistance under obese states by acting as a ligand for the cell surface GRP78/MTJ-1 complex in the liver.
- Kringle 5 is a ligand for the GRP78/VDAC complex, which increases intracellular Ca²⁺ concentration and induces apoptosis in endothelial cells.

**What New Information Does This Article Contribute?**

- Vaspin is a novel ligand for the cell-surface GRP78/VDAC complex in endothelial cells.
- Vaspin inhibits the kringle 5-induced Ca²⁺ influx and subsequent apoptosis in endothelial cells.

**Intimal proliferation in injured arteries is ameliorated in vaspin transgenic mice.**

Several adipokines play important roles in the development of vascular injuries in diabetes mellitus. This study finds that over-expression of vaspin ameliorates the intimal proliferation of arteries in mouse and rat diabetes mellitus models and vaspin inhibits the apoptosis of cultured endothelial cells by inhibiting kringle 5-induced Ca²⁺ influx. Our studies show the formation of a cell surface complex consisting of vaspin and GRP78/VDAC and that GRP78/VDAC is a vaspin receptor. Manipulation of the cell-surface GRP78/VDAC complex in endothelial cells by vaspin or other mimics represent a new therapeutic modality for the treatment of vascular diseases in diabetes mellitus.
Visceral Adipose Tissue-derived Serine Proteinase Inhibitor Inhibits Apoptosis of Endothelial Cells as a Ligand for the Cell-Surface GRP78/Voltage-dependent Anion Channel Complex

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http://circres.ahajournals.org/content/112/5/771

An erratum has been published regarding this article. Please see the attached page for:
/content/112/9/e98.full.pdf

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2013/01/10/CIRCRESAHA.111.300049.DC1

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In the *Circulation Research* article by Nakatsuka et al (Nakatsuka A, Wada J, Iseda I, et al. Visceral adipose tissue-derived serine proteinase inhibitor inhibits apoptosis of endothelial cells as a ligand for the cell-surface GRP78/voltage-dependent anion channel complex. *Circ Res.* 2013;112:771–780. DOI: 10.1161/CIRCRESAHA.111.300049), panels G and H in Figure 2 were inadvertently interchanged. The correct figure is as follows:

The error has been corrected in the online version of the article, which is available at http://circres.ahajournals.org/content/112/5/771.full.
Supplemental Material

Online Table I

<table>
<thead>
<tr>
<th>Gene Symbols</th>
<th>Forward primers</th>
<th>Reverse primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccl2 <em>(MCP-1)</em></td>
<td>5’-CAGGTCTCTGTACGCTTCT-3’</td>
<td>5’-TGTAAGTTCTCCAGCCGACTC-3’</td>
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<tr>
<td>Pdgfb</td>
<td>5’-CAAAGGAAGCAACCAGAAGTT-3’</td>
<td>5’-ACCATTGGCCGTCGAATCAG-3’</td>
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<tr>
<td>Pdgfrb</td>
<td>5’-TGCTCACAACGGTACCAAGATG-3’</td>
<td>5’-AATAGCACTTCGGGTCTAAATG-3’</td>
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<tr>
<td>Rplp0 <em>(36B4)</em></td>
<td>5’-GACAATGGCAGCAGTACAG-3’</td>
<td>5’-CAACAGTCGGGTAGC-3’</td>
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<tr>
<td>Serpina12 <em>(vaspin)</em></td>
<td>5’-AATGTGATGATGCGGACA-3’</td>
<td>5’-TACTGCCACCTGCCTCGAA-3’</td>
</tr>
</tbody>
</table>
Supplemental Figure I Balloon injuries of carotid arteries treated with adenovirus vector expressing LacZ (LacZ) and Vaspin (Vaspin-Ad) in streptozotocin-induced diabetic WKY rats. DeadEnd Colorimetric TUNEL system was used for Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. a and b, LacZ-treated carotid arteries. Apoptotic endothelial cell are seen (arrows). c and d, Apoptotic cells are reduced by the treatment of Vaspin-Ad. Bar = 100 μm in panels a and c, 50 μm in panels b and d. Insets in panels a and c correspond to panels b and d, respectively.
Supplemental Figure II Balloon injuries of carotid arteries treated with adenovirus vector expressing LacZ (LacZ) and Vaspin (Vaspin-Ad) in streptozotocin-induced diabetic WKY rats. Immunohistochemistry for GRP78 and VDAC. a and b, LacZ-treated carotid arteries. c and d, Vaspin-Ad treated carotid arteries. Bar = 50 μm.
Supplemental Figure III Human aortic endothelial cells (HAEC) culture using EGM-2 BulletKit treated with various concentrations of recombinant vaspin. 

(a) HAEC were treated with 25 mM D-glucose, which induced phosphorylation of eIF2α and IRE1α. Vaspin did not alter the phosphorylation of eIF2α and IRE1α. 

(b) 1.0 μg/ml tunicamycin and 5 μM thapsigargin induced phosphorylation of eIF2α and IRE1α, and up-regulated GRP78. Vaspin suppressed ER stress-related molecules induced by thapsigargin in a dose-dependent manner.
Supplemental Figure IV Human aortic endothelial cells (HAEC) culture using EGM-2 BulletKit in the presence of 5 μM thapsigargin. a, HAEC were transfected with 5 MOI (multiplicity of infection) of MISSION shRNA lentivirus transduction particles for GRP78 (NM_005347), VDAC (NM_003374) and Non-Target shRNA control lentivirus transduction particles, and they were further subjected to Western blot analysis of Akt and pAkt. b, CellTiter 96 Aqueous One Solution Cell Proliferation Assay. All data are presented as mean ± s.e.m. n = 4. *P < 0.05 vs. HAEC culture without Vaspin.
Supplemental Figure V Balloon injuries of carotid arteries treated with adenovirus vector expressing LacZ (LacZ) and Vaspin (Vaspin-Ad) in streptozotocin-induced diabetic WKY rats. Immunostaining for kringle 5 in a-d and pAkt in e and f. Bar = 100 μm in panels a and c, 50 μm in panels b, d, e, and f.
Supplemental Figure VI Schematic drawing of mechanism of vaspin action in endothelial cells.