Bile Acid Receptor TGR5 Agonism Induces NO Production and Reduces Monocyte Adhesion in Vascular Endothelial Cells

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Objective—TGR5 is a G-protein–coupled receptor for bile acids. So far, little is known about the function of TGR5 in vascular endothelial cells.

Approach and Results—In bovine aortic endothelial cells, treatment with a bile acid having a high affinity to TGR5, tauroliothocholic acid (TLCA), significantly increased NO production. This effect was abolished by small interfering RNA–mediated depletion of TGR5. TLCA-induced NO production was also observed in human umbilical vein endothelial cells measured via intracellular cGMP accumulation. TLCA increased endothelial NO synthase (eNOS) ser1177 phosphorylation in human umbilical vein endothelial cells. This response was accompanied by increased Akt ser473 phosphorylation and intracellular Ca²⁺. Inhibition of these signals significantly decreased TLCA-induced NO production. We next examined whether TGR5-mediated NO production affects inflammatory responses of endothelial cells. In human umbilical vein endothelial cells, TLCA significantly reduced tumor necrosis factor-α–induced adhesion of monocytes, vascular cell adhesion molecule-1 expression, and activation of nuclear factor-kB. TLCA also inhibited lipopolysaccharide-induced monocyte adhesion to mesenteric venules in vivo. These inhibitory effects of TLCA were abrogated by NO synthase inhibition.

Conclusions—TGR5 agonism induces NO production via Akt activation and intracellular Ca²⁺ increase in vascular endothelial cells, and this function inhibits monocyte adhesion in response to inflammatory stimuli. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: Akt • endothelial NO synthase • inflammation • intracellular Ca²⁺ • TGR5

NO produced by endothelial NO synthase (eNOS) is one of the critical signal molecules in the cardiovascular system. Besides its vasodilator effect, NO also has antiatherogenic properties, including inhibition of vascular smooth muscle cell proliferation and platelet aggregation, and monocyte-endothelial cells adhesion. NO production through eNOS activation is regulated by multiple mechanisms. The intracellular Ca²⁺ ([Ca²⁺]i) level is one of the most important factors. After a phospholipase C-γ–mediated [Ca²⁺]i increase, activated calmodulin (CaM) binds to the CaM-binding domain of eNOS, allowing allosteric activation. The pathway of phosphoinositide 3-kinase and its downstream serine/threonine kinase Akt is another important regulator. Activated Akt directly phosphorylates ser1177 on eNOS, which leads to increased sensitivity to Ca²⁺/CaM. Bile acids are products of cholesterol metabolism. Produced in the liver, bile acids circulate in the enterohepatic tract, which consists of the liver, gall bladder, bile duct, intestine, and portal vein. The classically known role of bile acids is to facilitate absorption of dietary lipids in the small intestine. Today, however, bile acids are highlighted as signal molecules rather than lipid solubilizers. It is well known that bile acids bind and activate several nuclear receptors, including the farnesoid X receptor. Farnesoid X receptor is highly expressed in the liver and intestine, regulating the transcription of specific target genes involved in bile acid, lipid, and glucose metabolism. Recent studies revealed that bile acids also serve as ligands for the G-protein–coupled receptor, TGR5 (or the G-protein–coupled bile acid receptor-1). TGR5 is expressed in various tissues and cell types, with relatively high expression in the gall bladder, intestine, placenta, and spleen. Previous in vitro studies showed that activation of TGR5 suppresses proinflammatory cytokine production and phagocytosis of monocytes/macrophages. Other in vivo studies broaden the immunosuppressive properties of TGR5 to include protective roles in metabolic diseases, such as atherosclerosis and diabetes mellitus, in which tissue infiltration and activation of immune cells is a hallmark of their initiation and development. Beneficial roles for TGR5 are also relevant in nonimmune cells. Stimulation of TGR5 leads to activation of thyroid hormone in brown adipose tissue and skeletal
Muscle\textsuperscript{11} and intestinal glucagon-like peptide-1 release,\textsuperscript{9} resulting in increased energy expenditure and improved glucose tolerance.

Because bile acids leak into the systemic blood flow from the enterohepatic tract, the vascular endothelium is constantly exposed to a certain amount of bile acids. Therefore, bile acid signaling may affect the physiological functions of endothelial cells. Indeed, farnesoid X receptor is expressed in vascular endothelial cells,\textsuperscript{12} and its activation leads to downregulation of endothelin-1 expression,\textsuperscript{13} upregulation of eNOS expression,\textsuperscript{14} and increased chemokinesis.\textsuperscript{15,16} However, the function of another bile acid receptor, TGR5, in endothelial cells remains poorly understood.

In this study, using bovine aortic endothelial cells and human umbilical vein endothelial cells (HUVECs), we demonstrate for the first time that TGR5 agonism induces NO production via Akt activation and [Ca\textsuperscript{2+}]\textsuperscript{2+} increase. Furthermore, we show that TGR5-mediated NO production can lead to reduced adhesion of monocytes both in vitro and in vivo.

### Materials and Methods

Materials and Methods are available in the online-only Supplement.

### Results

#### Vascular Endothelial Cells Express TGR5

In the preliminary experiments, we confirmed the expression of TGR5 protein in various types of vascular endothelial cells. As shown in Figure 1A, TGR5 protein expression was detected in all the types of endothelial cells we assessed, that is, bovine aortic endothelial cells, HUVECs, human pulmonary artery endothelial cells, and human dermal microvascular endothelial cells, using mouse gall bladder as a positive control.\textsuperscript{5} We also confirmed that small interfering RNA targeting TGR5 reduced its protein expression effectively in bovine aortic endothelial cells.

#### TGR5 Agonism Increased NO Production in Endothelial Cells

As shown in Figure 1B, treatment with a bile acid that has the highest affinity to TGR5, taurocholic acid (TLCA; 10–100 \textmu mol/L, for 30 minutes), dose-dependently increased the amount of NO produced in bovine aortic endothelial cells. Among other bile acids we tried, lithocholic acid (100 \textmu mol/L) also significantly increased NO production, but deoxycholic acid (100 \textmu mol/L) and chenodeoxycholic acid (100 \textmu mol/L) showed only marginal increases in NO production, which were not statistically significant. These results are in close agreement with previous reports showing that the order of increased cAMP production by various TGR5-mediated bile salts was TLCA > lithocholic acid > deoxycholic acid, \textsuperscript{7,17} a well-characterized purinergic activation of eNOS ATP-\gamma-S (30 \textmu mol/L) strongly increased NO production. We also confirmed that treatment with TLCA (100 \textmu mol/L, for 30 minutes) increased the intracellular level of cGMP in HUVECs, indicating NO production.

Pretreatment with N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA; 1 mmol/L) for 30 minutes abolished TLCA-induced cGMP accumulation (Figure 1C). Gene depletion of TGR5 abolished the TLCA-induced NO production, suggesting that this response is dependent on TGR5 (Figure 1D). There is a great deal of evidence showing that TGR5 agonism leads to increased intracellular cAMP.\textsuperscript{17,18} eNOS activity can be positively regulated by cAMP-dependent protein kinase A (PKA)–mediated phosphorylation.\textsuperscript{19,20} However, pretreatment with a PKA inhibitory peptide (30 \textmu mol/L) did not alter TLCA-induced NO production. Instead, TLCA (100 \textmu mol/L)-induced NO production was significantly inhibited...
TGR5 Agonism Increased eNOS<sup>ser1177</sup> Phosphorylation in Endothelial Cells

eNOS activity is positively regulated by phosphorylation of ser1177. In accordance with the results of NO measurement, treatment with TLCA (10–100 µmol/L) dose-dependently induced eNOS<sup>ser1177</sup> phosphorylation in HUVECs (Figure 2A). The TLCA (100 µmol/L)-induced phosphorylation peaked 10 to 20 minutes after initiation (Figure 2B). As expected, pretreatment with a calcium channel blocker, La<sup>3+</sup> (300 µmol/L) or LY294002 (10 µmol/L), significantly reduced TLCA (100 µmol/L)-induced eNOS<sup>ser1177</sup> phosphorylation (Figure 2C). We also assessed TGR5-mediated changes in phosphorylation state of thr495, which negatively correlates with the enzyme activity. Nevertheless, TLCA (100 µmol/L, for 2–20 minutes) did not significantly alter the phosphorylation level of eNOS<sup>thr495</sup> in HUVECs (n=4). This result suggests that TGR5 agonism exerts little effect on intracellular signals, which regulate eNOS<sup>thr495</sup> phosphorylation, such as protein kinase C, protein phosphatase 2A, or protein phosphatase 1.

TGR5 Agonism Increased Akt Ser473 Phosphorylation in Endothelial Cells

We next examined whether TGR5 agonism activates the phosphoinositide 3-kinase/Akt pathway. As shown in Figure 3A, treatment with TLCA (100 µmol/L) induced Akt<sup>ser473</sup> phosphorylation in HUVECs with the peak occurring 10 minutes after initiation. Pretreatment with LY294002 completely abolished the Akt<sup>ser473</sup> phosphorylation in both the resting and the TLCA (100 µmol/L)-stimulated states. Pretreatment with La<sup>3+</sup> (300 µmol/L) also significantly reduced TLCA-induced Akt<sup>ser473</sup> phosphorylation (Figure 3B). This result is in agreement with previous reports showing deprivation of extracellular Ca<sup>2+</sup> inhibited both Akt and eNOS activation induced by vascular endothelial growth factor or sphingosine-1-phosphate. As in the case of NO production, Akt<sup>ser473</sup> phosphorylation was also induced by lithocholic acid but not by deoxycholic acid or chenodeoxycholic acid (Figure 3C).

TGR5 Agonism Increased [Ca<sup>2+</sup>]<sub>i</sub> in Endothelial Cells

We next examined the changes in [Ca<sup>2+</sup>]<sub>i</sub>, induced by bile acid/TGR5 signaling using a calcium indicator, fura-2 AM. As shown in Figure 4A and 4B, treatment with TLCA (10–100 µmol/L) or a positive control, ATP-γS (30 µmol/L), rapidly increased [Ca<sup>2+</sup>]<sub>i</sub> of HUVECs in a dose-dependent manner. This effect was abolished by pretreatment with La<sup>3+</sup> (300 µmol/L) or U-73122 (3 µmol/L). [Ca<sup>2+</sup>]<sub>i</sub> also was significantly increased by lithocholic acid but not by deoxycholic acid or chenodeoxycholic acid. The receptor-induced [Ca<sup>2+</sup>]<sub>i</sub>-mobilization involves the rapid, transient release of Ca<sup>2+</sup> from stores, mainly the endoplasmic reticulum followed by sustained entry of extracellular Ca<sup>2+</sup>. As shown in Figure 1 in the online-only Data Supplement, in the absence of extracellular Ca<sup>2+</sup> treatment with a TGR5 agonist, TLCA induced a sustained Ca<sup>2+</sup> entry. In the presence of La<sup>3+</sup> (300 µmol/L), TLCA-induced Ca<sup>2+</sup> entry was abolished. Collectively, TGR5-mediated [Ca<sup>2+</sup>]<sub>i</sub> increase was attributable to initial phospholipase C-γ-dependent Ca<sup>2+</sup> release from endoplasmic reticulum and after extracellular Ca<sup>2+</sup> entry.

TGR5 Agonism Reduces Tumor Necrosis Factor-α-Induced Vascular Cell Adhesion Molecule-1 Expression in Endothelial Cells and Monocyte Adhesion

Adhesion of monocytes to endothelial cells via adhesion molecules is a key step in the development of atherosclerosis. NO is known to suppress the expression of adhesion molecules induced by inflammation. Whether TGR5-mediated NO production affects the inflammatory response of endothelial cells was assessed. As shown in Figure 5A, HUVECs stimulated by tumor necrosis factor (TNF)-α (1 ng/mL; 6 hours) displayed significantly increased adhesion to the human monocyte cell line, U937. This response was significantly inhibited when HUVECs were concurrently treated with TLCA (100 µmol/L). Both Western blot analysis and cell-based ELISA revealed that treatment with TNF-α increased the expression of an adhesion molecule, vascular cell adhesion...
molecule-1 (VCAM-1). This effect of TNF-α was repressed by the treatment with TLCA (Figure 5B and 5C). As shown in Figure 5D, the p65 subunit of a pivotal proinflammatory transcription factor, nuclear factor (NF)-κB, is widely distributed in the cytoplasmic region of quiescent cells as well as cells treated with TLCA alone (data not shown). However, stimulation with TNF-α induced translocation of NF-κB p65 to the nuclei in almost all cells indicating its activation. When treated with both TNF-α and TLCA, NF-κB p65 was still detected in the nuclear region of some cells, but overall was found to a greater degree in the cytoplasmic region. Treatment with L-NMMA (1 mmol/L) 30 minutes before administration of TLCA abrogated its inhibitory effect on U937 adhesion (Figure 5A), VCAM-1 upregulation (Figure 5B and 5C), and NF-κB p65 nuclear translocation (Figure 5D) all induced by TNF-α.

TGR5 Agonism Reduces Lipopolysaccharide-Induced Monocyte Adhesion to Vascular Wall In Vivo

Finally, we investigated whether TGR5 agonism inhibits monocyte adhesion to endothelial cells via NO production in vivo. As shown in Figure 6A, intraperitoneal administration of lipopolysaccharide (1 mg/kg, 6 hours) significantly increased the number of monocytes adhered to the vascular wall of mesenteric venules. Concurrent administration of TLCA (3 mg/kg) significantly inhibited the monocyte adhesion. The inhibitory effect of TLCA was hampered by pretreatment with a NOS inhibitor, L-NMMA (15 mg/kg, 30 minutes before TLCA treatment). Consistently, 2 hours after administration of lipopolysaccharide, mRNA expression of VCAM-1, as well as another adhesion molecule, intercellular adhesion molecule-1, was significantly increased in the mesenteric vessel. Administration of TLCA significantly attenuated expression of these adhesion molecules induced by lipopolysaccharide. This inhibition was abrogated by pretreatment with L-NMMA (Figure 6B). In line with the observation in vitro, these in vivo results also indicate that TGR5-mediated NO production can inhibit adhesion molecule expression in endothelial cells and subsequent monocyte adhesion.

Discussion

In the present study, we demonstrated for the first time that TGR5 signaling induces NO production in vascular endothelial cells. This reaction is dependent on [Ca]i increase and Akt activation. TGR5-mediated NO production can suppress the expression of an adhesion molecule and subsequent adhesion of monocytes to endothelial cells via inhibition of NF-κB activity.

It is widely recognized that a Gαs/cAMP signal pathway is associated with a variety of biological functions induced by TGR5 agonism in many types of cells.7–10,17 In the context of NO production, Keitel et al18 demonstrated that TGR5 agonism in rat liver sinusoidal endothelial cells induced eNOS phosphorylation, which is accompanied by intracellular cAMP elevation. Because eNOS activity is reported to be regulated by PKA-mediated phosphorylation,19,20 TGR5-mediated cAMP accumulation may activate eNOS. In the present study, however, pretreatment with PKA inhibitory peptide failed to reduce TGR5-mediated NO production, suggesting that cAMP/PKA is not involved in the response.
our results suggest that TGR5-mediated NO production in endothelial cells is dependent on $[\text{Ca}]^{2+}$ increase and Akt activation. Hong et al.\(^{25}\) also found a cAMP/PKA-independent biological response mediated by TGR5, in which stimulation of TGR5 leads to G\(_{\alpha q}\)-dependent H\(_2\)O\(_2\) production and cell proliferation in esophageal adenocarcinoma.\(^{25}\) More detailed investigations are needed to elucidate the intracellular signals lying downstream of TGR5.

Although NO is considered to be a key antiatherogenic molecule produced by endothelial cells, it is not completely understood how NO suppresses the expression of adhesion molecules. Our results suggest that inhibition of NF-\(\kappa\)B activity by TGR5-mediated NO production is responsible for the suppression of adhesion molecule expression. Previous reports also suggest that NO inhibits NF-\(\kappa\)B activity, which is dependent on induction and nuclear translocation of inhibitor \(\kappa\)B.\(^{24,26}\) Another report suggested that NO directly inhibits DNA binding activity of NF-\(\kappa\)B through S-nitrosylation of certain cysteine residues.\(^{27}\) Further studies are required for a better understanding of how TGR5-mediated NO regulates adhesion molecule expression in endothelial cells.

A growing body of evidence suggests that TGR5 is an attractive target of treatment for metabolic diseases, including atherosclerosis. A previous study demonstrated that oral administration of a TGR5 agonist, INT-777, inhibited atherosclerotic lesion formation in a well-established genetic animal model for atherosclerosis using low-density lipoprotein receptor–deficient mice. This effect was associated with reduced intraplaque macrophage content and proinflammatory cytokine production. However, in low-density lipoprotein receptor–deficient TGR5\(^{-/-}\) double-knockout mice, no significant effect was seen with the agonist. The authors also showed that stimulation of TGR5 reduced the release of proinflammatory cytokines and the uptake of oxidized low-density lipoprotein in macrophages in vitro.\(^{28}\) In the pathophysiology of atherosclerosis, these actions by macrophages are preceded by the expression of adhesion molecules in inflamed endothelial cells. This leads to the recruitment of circulating monocytes, which are then transformed into macrophages. Therefore, understanding how TGR5 signaling modulates the inflammatory response of endothelial cells is also important for developing the treatment for atherosclerosis targeting TGR5.
In this respect, the present study suggests that TGR5 may also play atheroprotective roles in vascular endothelial cells and supports the idea that TGR5 is a valuable candidate for the prevention, as well as treatment, of atherosclerosis.

In healthy subjects, the serum concentration of bile acids fluctuates from ≈2 μmol/L in the fasting state to ≈15 μmol/L after a meal. With this variation, serum bile acids may be able to hormonally act on endothelial cells and regulate their function by reflecting nutrient uptake. In the case of patients with liver diseases, such as cirrhosis, the serum bile acid concentration by reflecting nutrient uptake. In the case of patients with liver diseases, such as cirrhosis, the serum bile acid concentration is elevated drastically, often exceeding 100 μmol/L.29,30 This abnormally high level of bile acids may induce the pathophysiological changes of endothelial cells associated with hepatic diseases. It is not yet clear which biological phenomena in vivo TGR5-mediated NO production is most related to and, therefore, warrants further study.

In conclusion, we show that TGR5 agonism induces NO production in vascular endothelial cells, and that this leads to suppression of VCAM-1 expression and monocyte adhesion. The current report sheds light on why TGR5 should be considered as a potential new candidate to modulate the physiological and pathophysiological functions of vascular endothelial cells.

**Sources of Funding**
This work was supported by a Grant-in-Aid for JSPS Fellows to T. Kida and a Grant-in-Aid for Young Scientists (A) and Grant-in-Aid for Challenging Exploratory Research from the Japan Society for the Promotion of Science, The Naito Foundation, The Takeda Science Foundation, The SENSHIN Medical Research Foundation, and the Japan Diabetes Foundation to T. Murata.

**Disclosures**
None.

**References**
TGR5 is a G-protein–coupled receptor for bile acids, and is expressed in various tissues and cell types. Because TGR5 is known to improve glycemic control and enhance energy expenditure in response to ligand activation, it is considered to be an attractive target of treatments for metabolic diseases. However, little is known about the function of TGR5 in the vasculature. Because bile acids leak into the systemic blood flow from the enterohepatic circulation, we assume that bile acids can act on endothelial cells to modulate their physiological functions via TGR5. We here demonstrated that TGR5 agonism induces NO production via intracellular Ca++ increase and Akt activation in vascular endothelial cells, and this leads to suppression of monocyte adhesion. This report highlights vasoprotective properties of bile acids through TGR5 activation for the first time, and would shed light on TGR5 as a potential target for cardiovascular diseases.
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Arterioscler Thromb Vasc Biol. published online April 25, 2013;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Materials and Methods

Chemicals

The chemicals used were as follows: taurolithocholic acid (TLCA), L-arginine, tetrahydrobiopterin, ionomycin, U73122, chenodeoxycholic acid (CDCA), thapsigargin, lipopolysaccharide (LPS) from Escherichia coli O55:K5 (Sigma, MO), penicillin-streptomycin (Invitrogen, CA), deoxycholic acid (DCA), lithocholic acid (LCA), N\(^6\)-monomethyl-L-arginine (L-NMMA), 3-isobutyl-1-methylxanthine (IBMX) (Wako Pure Chemical, Japan), lanthanum chloride (LaCl\(_3\)) (Nacalai Tesque, Japan), Pefabloc SC, complete protease inhibitor cocktail tablets (Roche Diagnostics, Switzerland), fura 2-AM (Dojindo Laboratories, Japan), PKA Inhibitor 14-22 Amide, Cell-Permeable, Myristoylated (Merk KGaA, Germany)

Cell culture procedure

HUVECs (Lonza, MD) were cultured in EGM-2 Bullet Kit medium (Lonza) containing growth supplements. Confluent HUVECs were used at passages 2-7 after starvation with EBM-2 containing no growth supplements for 4 h. BAECs were isolated from bovine thoracic aortae, and cultured in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Confluent BAECs were used at passages 3-8 after starvation with FBS-free DMEM for 16-24 h. For gene depletion of TGR5, BAECs at 40-60% confluence were transfected with a set of RNAi\(^\text{TM}\) siRNAs targeting TGR5 (GPBAR1-HSS152622, GPBAR1-HSS152623, GPBAR1-HSS152624, Invitrogen, 25 nM each), using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer’s instruction. Cells were used for experiments at least 90 h after the transfection.

Measurement of NO production

BAECs were washed with HEPES-buffered solution, and incubated in HEPES-buffered solution supplemented with 1mmol/L L-arginine and 10 μmol/L tetrahydrobiopterin. After equilibrating for 30 min, media were changed and cells were incubated for another 30 min. Subsequently, cells were stimulated with the indicated agents for 30 min. Conditioned media were collected, and centrifuged at 300 × g for 5 min. The supernatants were used for measurement of nitrite and nitrate, stable metabolites of NO, by ENO-20 NOx analyzer (Eicom, Japan). The increased nitrite and nitrate levels after stimulation were normalized to the cell protein content.
Measurement of cGMP accumulation

HUVECs were pretreated with a phosphodiesterase inhibitor, IBMX (200 μmol/L) for 5 min. After stimulation, cells were lysed and the cGMP concentration in the lysate was measured by enzymeimmunoassay (cAMP Biotrak Enzymeimmunoassay system, GE Healthcare, UK).

Western blotting

Protein samples were extracted from HUVECs using the following lysis buffer: 50 mmol/L Tris-HCl (pH 7.4), 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1% NP-40, 0.1% SDS, 0.1% sodium deoxycholate, 50 mmol/L NaF, 1 mmol/L Na3VO4, and 10 mmol/L β-glycerophosphate. Pefabloc SC (1.0 mg/ml) and complete protease inhibitor cocktail tablets (1 tablet/50 ml) were added before use. 30 μg protein samples were loaded on 7.5-12.5% polyacrylamide gel, and separated by SDS-PAGE. Protein-blotted polyvinylidine difluoride (PVDF) membranes were blocked in TBS containing 3% bovine serum albumin (BSA) and subsequently incubated with following primary antibodies: mouse monoclonal anti-phospho-eNOS (ser1177), mouse monoclonal anti-eNOS, rabbit polyclonal anti-Akt (BD Transduction Laboratories, NJ; all 1000× dilution), rabbit polyclonal anti-phospho-eNOS (thr495) (Millipore, MA; 500× dilution), mouse monoclonal anti-β-actin (Sigma, MO; 1000× dilution), mouse monoclonal anti-phospho-Akt (Ser473) (Cell Signaling, MA; 500× dilution), rabbit polyclonal anti-TGR5 (Abcam, MA; 500× dilution), goat polyclonal anti-VCAM-1 (Santa Cruz Biotechnology, CA; 300× dilution). Membranes were then incubated with the following secondary antibodies where appropriate: IRDye 680 goat anti-rabbit antibody, IRDye 800CW goat anti-mouse antibody, IRDye 800 donkey anti-goat antibody (LI-COR Biosciences, NE, all 10000× dilution). Membranes were scanned, and the optical density of each band was quantified using an Odyssey infrared imaging system (LI-COR Biosciences).

Measurement of intracellular Ca2+ concentration

HUVECs cultured on glass coverslips were stained with the fluorescent Ca2+ probe, fura 2-AM (3 μmol/L) with 0.01% cremophor EL in DMEM for 30 min. The cells were washed with HEPES-buffered saline. The coverslips with the stained cells were mounted on slides and placed in an open chamber on a fluorescent microscope (Eclipse TE300, Nikon, Japan). Changes in the fluorescent intensity at 340 and 380 nm (F340 and F380) and the fluorescence ratio (F340/F380) were measured every 3 seconds with a fluorescence imaging system (Hamamatsu Photonics, Japan). To normalize the
data, at the end of every experiment, the fluorescence ratios both in the presence of 1 μmol/L ionomycin with Ca\(^{2+}\)-free HEPES-buffered saline and in normal HEPES-buffered saline were measured.

**Cell-adhesion assay**

Confluent HUVECs were stimulated with tumor necrosis factor (TNF)-α (1 ng/ml) for 6 h. After washing, 2.5×10\(^5\) U937 cells were layered onto a stimulated endothelial monolayer and incubated for 1 h. Non-adherent cells were then removed by washing and the remaining cells were fixed with 4% paraformaldehyde for 5 min. The number of U937 cells was then counted in randomly-selected 5 fields using an inverted microscope (Eclipse ES100, Nikon).

**Cell-based ELISA**

Confluent HUVECs in 96-well plates were stimulated in the same way as in cell-adhesion assay. The cells were fixed with 4% paraformaldehyde for 5 min. After washing twice with PBS, the cells were permeabilized with 0.5% tween-20 for 5 min, followed by blocking with 3% BSA for 1 h. Then the cells were incubated with goat polyclonal anti-VCAM-1 antibody (Santa Cruz Biotechnology, 100× dilution) overnight. Negative controls were obtained by omitting primary antibody in this step. After washing twice with PBS, the cells were incubated with IRDye 800 donkey anti-goat antibody (LI-COR Biosciences, 10000× dilution) for 1 h. Plates were scanned, and the optical density of each well was quantified using an Odyssey infrared imaging system (LI-COR Biosciences). The optical density of the negative control was subtracted as background from that of each well.

**Immunostaining**

HUVECs on gelatin-coated sterilized glass coverslips were fixed with 4% paraformaldehyde for 5 min. After washing twice with PBS, the cells were permeabilized with 0.2% Triton X-100 for 30 min, followed by blocking with 3% BSA for 1 h. Then the cells were incubated with rabbit polyclonal anti-NF-κB p65 antibody and goat polyclonal anti-VE-cadherin antibody (Santa Cruz Biotechnology, 100× and 250× dilutions, respectively) overnight. After washing twice with PBS, the cells were incubated with Alexa Fluor 594-conjugated donkey anti-rabbit IgG antibody and Alexa Fluor 488-conjugated donkey anti-goat IgG antibody (Invitrogen, 300× dilution) for 3 h. The cells were finally incubated with 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/mL) to stain the nuclei and photographed using a fluorescence microscope (Eclipse E800,
Intravital microscopy of mouse mesenteric venules

C57BL/6 mice were intraperitoneally administered with each agent. After 6 h, mice were anesthetized and kept at 37°C on a heating pad. To visualize the blood vessel, the mice were intravenously injected with fluorescein isothiocyanate (FITC)-dextran, and then the mesentery was gently exteriorized and prepared for intravital microscopy. Monocytes adhered to the vessel wall were counted in at least 3 venular areas with the diameter of ~50 µm and length of ~100 µm using a confocal microscope (Eclipse Ti, Nikon) equipped with an argon laser.

Quantitative RT-PCR

Total RNA was extracted from mesenteric artery and vein of mice 2 h after intraperitoneal administration of each agent. The first strand of cDNA was synthesized using random 9 mers RT-primer and ReverTra Ace (Takara Bio, Japan). Real time PCR was performed over 45 cycles on ABI Prism 7000 (Applied Biosystems, CA) using SYBR Green (Invitrogen) as a reporter dye. Primers used were as follows: 18S rRNA, GACTCAACACGGGAACCTCAC (forward) and CACCCACGGAATCGAGAAAG (reverse); VCAM-1, TACCAGCTCCCCAAAATCCTG (forward) and TCTGCTAATTCCAGCCTCGT (reverse); ICAM-1, TTCACACTGAATGCCAGCTC (forward) and GTCTGCTGAGACCCCTCTTG (reverse). The expression abundance was determined by the ΔCt method, using 18S rRNA as an endogenous control gene.

Statistical analysis

The results are expressed as means ± S.E. Statistical evaluation of the data was performed by one-way analysis of variance followed by Bonferroni’s test. A p value <0.05 was considered significant.