Withaferin A is an inhibitor of endothelial protein C receptor shedding in vitro and in vivo

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ABSTRACT

Withaferin A (WFA), an active compound from Withania somnifera, has been widely researched for its anti-inflammatory and cardioactive properties and effects on the central nervous system. The endothelial cell protein C receptor (EPCR) plays important roles in blood coagulation and inflammation. EPCR activity is markedly changed by ectodomain cleavage and release as the soluble EPCR. EPCR is shed from the cell surface, mediated by tumor necrosis factor-α converting enzyme (TACE). In this study, we investigated the effects of WFA on the EPCR shedding in human umbilical vein endothelial cells (HUVECs) and in mice and the associated signaling pathways. WFA was found to induce inhibition of phospholipid-12-myristate 13-acetate (PMA), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and on cecal ligation and puncture (CLP)-induced EPCR shedding and WFA suppressed the expression and activity of TACE. In addition, treatment with WFA resulted in reduced PMA-stimulated phosphorylation of p38, extracellular regulated kinases (ERK) 1/2, and c-Jun N-terminal kinase (JNK). These results demonstrate a therapeutic potentiality of WFA as an anti-sEPCR shedding reagent against PMA and CLP-mediated EPCR shedding. © 2014 Elsevier Ltd. All rights reserved.

1. Introduction

The protein C (PC) system is an important natural anticoagulant mechanism, involving proteolytic degradation of the procoagulant cofactors factor Va and VIIIa, through activated protein C (APC) (Fulcher et al., 1984). PC, a vitamin K-dependent zymogen, is activated at the endothelial surface when thrombin binds to thrombomodulin, a protein that transforms the procoagulant enzyme into a potent activator of PC (Mosnier et al., 2007). Another endothelial factor that contributes to the PC anticoagulant pathway is the endothelial cell protein C receptor (EPCR) (Fukudome and Esmon, 1994). This receptor, which can bind to PC or APC with the same affinity (dissociation constant \( K_d = 30 \text{ nM} \)), is mainly expressed on endothelial cells of large vessels (Mosnier et al., 2007). EPCR is a 46-kDa type 1 transmembrane glycoprotein homologous to major histocompatibility complex class I family proteins (Fukudome and Esmon, 1994; Villoutreix et al., 1999). This 221-amino-acid (aa) protein comprises an extracellular domain, a 25-aa transmembrane domain, and a short (3 aa) intracytoplasmic sequence (Fukudome and Esmon, 1994; Villoutreix et al., 1999).

The gene is located on chromosome 20 (Hayashi et al., 1999), it spans 8 kilobase (kb) and comprises 4 exons (Hayashi et al., 1999; Simmonds and Lane, 1999). A soluble form of EPCR (sEPCR) is generated from the membrane bound EPCR through proteolytic cleavage by metalloproteinase activity that can be induced by thrombin and other inflammatory mediators (Xu et al., 2000), a process that is called shedding. sEPCR circulates in plasma and retains its ability to bind both PC and APC but does not enhance protein C activation (Kurosawa et al., 1997). It inhibits APC anticoagulant activity by forming a complex that involves phospholipid membranes (Liaw et al., 2000). sEPCR resulting from shedding of membrane EPCR, can be detected in plasma concentration of approximately 100 ng/m; high levels of sEPCR have been reportedly associated with systemic inflammatory diseases (Kurosawa et al., 1998). In vitro studies have described a dramatic increase in EPCR shedding from the endothelium by a wide variety of inflammatory mediators (IL-1β, H₂O₂, and phospholipid myristate acetate) and thrombin, and EPCR shedding is potentiated by the microtubule disrupting agent, nocodazole (Xu et al., 2000). In addition, phosphorylation of mitogen-activated protein (MAP) kinases such as p38, ERK1/2, and JNK were shown to be increased by stimulation with PMA (Han et al., 2010; Leng et al., 2004; Menschikowski et al., 2009) and activation sequence (Fukudome and Esmon, 1994; Villoutreix et al., 1999).
of TACE was shown to occur upon activation of ERK or p38 (Huovila et al., 2005; Murphy, 2008). Therefore, the shedding of EPCR might be connected with the activation of MAPK or TACE directly or indirectly.

The search for anticancer drugs and anti-inflammatory agents from natural products represents an area of interest worldwide (Aggarwal et al., 2006). *Withania somnifera* has been used to prevent infections of burns, wounds, and dermatological disorders (Essawi and Srour, 2000). Withaferin A (WFA, Fig. 1) is a steroidal lactone derived from *W. somnifera*, a plant that has been used for centuries to treat several inflammatory disorders (Kaileh et al., 2007). However, the effect of WFA on EPCR shedding and its underlying mechanisms in both cellular system and animal model have not yet been elucidated. Anti-inflammatory effects of WFA in HUVECs and mice have recently been reported (Lee et al., 2012). Previous report showed that PMA-stimulated EPCR shedding is mediated by tumor necrosis factor-α converting enzyme/ADAM17 (TACE) (Qu et al., 2007). Noting that sEPCR serves as a marker of vascular barrier integrity in vascular inflammatory disease, and sEPCR is involved in the pathophysiology of sepsis (Borgel et al., 2007; Kurosawa et al., 1998); we hypothesized that WFA may have anti-sEPCR shedding activity. Therefore, in the present study, we investigated the effect of WFA on the expression and activity of TACE and against PMA-induced EPCR shedding in human endothelial cells and in a cecal ligation and puncture (CLP) model of sepsis in mice.

2. Materials and methods

2.1. Reagents

WFA (Fig. 1) was purchased from Biomol (Plymouth Meeting, PA, USA). sEPCR and TNF-α were purchased from Abnova (Taipei City, Taiwan). Phorbol-12-myristate 13-acetate (PMA) and IL-1β were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex Bio Science (Charles City, IA) and maintained as previously described (Bae and Rezaie, 2011). HUVECs of passage numbers 3 or 4 were used in the experiments.

2.3. Animals and husbandry

Male C57BL/6 mice (6–7 weeks old, weighting 18–20 g) were purchased from Orient Bio Co. (Sungnam, KyungKido, Korea), and used after a 12-day acclimatization period. Five animals per polycarbonate cage were housed under controlled conditions (20–25 °C/RH 40–45%) under a 12-h light/dark cycle, and supplied a normal rodent pellet diet and water ad libitum. All animals were treated in accordance with the Guidelines for the Care and Use of Laboratory Animals issued by Kyungpook National University.

2.4. Enzyme-linked immunosorbent assay (ELISA) for cellular EPCR expression

Modified whole-cell ELISA was performed as previously described to determine expression levels of EPCR on HUVECs (Kim et al., 2011; Lee et al., 2013). Briefly, confluent monolayers of HUVECs were treated with or without WFA for 6 h, followed by treatment with PMA, tumor necrosis factor (TNF)-α, or interleukin (IL)-1β for 1 h. Media were then removed, and cells were washed with PBS and fixed with 50 μl of 1% paraformaldehyde for 15 min at room temperature. After washing, 100 μl of rabbit polyclonal EPCR antibodies (Abnova, Taipei City, Taiwan) was added, and 1 h (37 °C, 5% CO2) later, cells were washed 3 times, followed by treatment with 100 μl of 1:2000 peroxidase-conjugated anti-rabbit IgG antibodies (Sigma, St. Louis, MO, USA) for 1 h. Cells were then washed 3 times and developed using o-phenylenediamine substrate (Sigma, St. Louis, MO, USA). Colorimetric analysis was performed by measurement of absorbance at 490 nm. All measurements were performed in triplicate wells.

2.5. Competitive competitive ELISA for sEPCR and TACE

Ninety-six-well flat microtiter plates (Corning, NY) were coated overnight at 4 °C with sEPCR protein for determination of sEPCR or TACE protein for determination of TACE in 20 mM carbonate–bicarbonate buffer (pH 9.6) containing 0.02% sodium azide. Lyophilized culture media were prepared for sEPCR, and total cell lysates were prepared for TACE using lysis buffer containing (mM): Tris–HCl (20) pH 7.5, EGTA (0.5), EDTA (2), diithiothreitol (2), p-methylsulfonyl fluoride (0.5), and 10 μg/ml leupeptin. Plates were then rinsed 3 times in PBS-T and kept at 4 °C. Prepared samples from cell culture media and mice plasma for sEPCR or from cell lysates for TACE were pre-incubated with anti-EPCR antibodies (rabbit polyclonal, 1:500, Abnova, Taipei City, Taiwan) or anti-TACE antibodies (goat polyclonal, 1:500, Santa Cruz, Dallas, Texas, USA) in 96-well plastic round microtiter plates for 90 min at 37 °C, transferred to pre-coated plates, and incubated for 30 min at room temperature. Plates were then rinsed 3 times with PBS-T, incubated for 90 min at room temperature with peroxidase-conjugated anti-rabbit or anti-goat IgG antibodies (1:2000, Amersham Pharmacia Biotech, Uppsala Sweden), rinsed three times in PBS-T, and incubated for 60 min at room temperature in the dark with 200 μl of substrate solution (100 μg/ml o-phenylenediamine containing 0.003% H2O2). The reaction was then stopped by the addition of 50 μl of 8 N H2SO4, and absorbances at 490 nm were measured.

2.6. TACE activity assay

For TACE activity assay, commercially available TACE activity kit (Innozyme TACE activity assay kit, EMD Millipore, Billerica, MA, USA) was used as described previously (Miller et al., 2013). Confluent monolayers of HUVECs in 12-well culture plates were treated with or without WFA for 6 h, followed by treatment with PMA (1 μM) for 1 h. Media were then removed, and total cell lysates were prepared using the CytoBuster™ Protein Extraction Reagent (EMD Millipore, Darmstadt, Germany). Total cell lysates were applied to plates pre-coated with a monoclonal antibody specific for human TACE. TACE activity was measured using an internally quenched fluorescent substrate, MCA-KPLG-Dpa-AR-NH2. The cleavage rates were detected within the dynamic range of the assay according to a standard curve.

2.7. Cecal ligation and puncture (CLP)

For induction of sepsis, male mice were anesthetized with zoletil 50 and rompun. The CLP-induced sepsis model was prepared as previously described (Wang et al., 2004). In brief, a 2 cm midline incision was made in order to allow exposure of the cecum and adjoining intestine. The cecum was then tightly ligated using a 3.0-silk suture (SK34510, Ailee, South Korea) at 50 mm from the cecal tip, punctured once with a 22-gauge needle, gently squeezed in order to extrude a small amount of feces, and returned to the peritoneal cavity. The laparotomy site was closed using a horizontal continuous suture with 5-0 polyglactin-910. After 12 h or/and 50 h CLP surgery, WFA (1.882 μg/mouse) was administrated intravenously. In each day after CLP, blood was collected via intravenously. Or, after 4 days CLP, mice was sacrificed by cervical dislocation for immunohistochemistry. This protocol was approved in advance by the Animal Care Committee at Kyungpook National University (KNU 2012–13).

2.8. Immunohistochemistry

To observe the expression pattern of EPCR, aortas from CLP-induced septic (Day 4) and sham operated mice were removed and fixed in 4% formaldehyde solution (Junes, Japan) in PBS for 20 h at 4 °C. After fixation, the aortas were dehydrated through an ethanol series, embedded in paraffin, and cut into 3 μm sections. Depa- raffinized sections were quenched in 3% H2O2 in methanol; washed in PBS; placed in boiled 1 mM Tris solution (pH 9.0), supplemented with 0.5 mM EGTA solution in order to reveal the antigens; and blocked for 1 h at RT in PBS, supplemented with 1% bovine serum albumin, 0.2% gelatin, and 0.05% saponin. Sections were incubated with anti-rabbit EPCR antibody (Alcam, Cambridge, MA, USA) diluted 1:500 in PBS, and supplemented with 0.1% BSA and 0.3% Triton X 100 for 1 h at 4 °C in a humidified chamber. After washing in PBS, supplemented with 0.1% BSA, 0.2% gelatin, and 0.05% saponin, the sections were incubated with peroxidase-conjugated anti-rabbit IgG antibody (DAKO, Glostrup, Denmark) for 1 h at RT and then developed using the Liquid DAB + Substrate-Chromogen System (DAKO, Glostrup, Den-
Table 1  
Pharmacokinetic parameters of WFA after administration of 5 mg/kg (n = 5).  

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Intravenous injection</th>
</tr>
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<tbody>
<tr>
<td>AUC*</td>
<td>8.4 μg h/ml</td>
</tr>
<tr>
<td>Half-life ((t_{1/2}))</td>
<td>1.9 h</td>
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<tr>
<td>(C_{\text{max}})</td>
<td>3.69 μg/ml (7.83 μM)</td>
</tr>
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* AUC = area under the curve.

2.10. Pharmacokinetic parameters

After an intravenous penile vein injection of male C57BL/6 mice with WFA at 5 mg/kg, dosing was performed with 0.5% methyl green in ddH2O. Non-immune rabbit IgG (DAKO, at the same concentration instead of EPCR antibody) and anti-rabbit CD31 antibody (1:200, Abcam, Cambridge, MA, USA) was used as negative and positive control for immunohistochemistry, respectively.

2.9. ELISA for total and phospho p-38MAPK, ERK1/2, and JNK

HUVECs were cultured in 96-well microtiter plates for quantitative determination of p38MAPK, ERK1/2, and JNK phosphorylation. On the day of experiments, culture medium was replaced by serum-free growth medium. Cells were then treated with or without WFA for 6 h, followed by treatment with PMA (1 μM) for 1 h. Activation of p38MAPK, ERK 1/2, and JNK was quantified in nuclear lysates using ELISA kits for total/phosphorylated p38MAPK (Life Technologies, Carlsbad, California, for total p38MAPK or Cell Signaling Technology, Danvers, MA, USA, for phosphorylated-p38MAPK), total/phospho ERK1/2 and JNK (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

2.11. Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM) of at least three independent experiments with duplicate determination. Statistical significance was defined to be based on a p value smaller than 0.05 (SPSS, version 14.0, SPSS Science, Chicago, IL, USA).

3. Results and discussion

In this study, the effects of WFA (Fig. 1) on the shedding of EPCR were determined in vitro and in vivo. The pharmacokinetic values in plasma of WFA are shown in Table 1.

3.1. Effect of WFA on PMN, TNF-α, or IL-1β-induced EPCR shedding

Previous studies have reported that PMA stimulates EPCR shedding from HUVECs (Qu et al., 2006, 2007). In agreement with the previous results, we found that as little as 0.1 μM PMA (Fig. 2A and B) could fully stimulate EPCR shedding from HUVECs (Fig. 2A) and that cellular EPCR on HUVECs showed a PMA-mediated dose-dependent decrease (Fig. 2B). EPCR shedding by TNF-α or interleukin (IL)-1β also showed an increase (Fig. 2C and D), in agreement with a previous study (Menschikowski et al., 2009).

To investigate the effect of WFA on PMA-mediated EPCR shedding, endothelial cells were pretreated with increasing concentrations of WFA for 6 h, followed by stimulation with 1 μM PMA for 1 h. As shown in Fig. 3A and B, treatment with WFA resulted in inhibition of EPCR shedding induced by PMA in endothelial cells, with an optimal effect at 1–2 μM. WFA alone (2 μM) did not affect
the shedding of EPCR. Therefore, WFA alone (2 μM) did not affect the total levels of membrane bound EPCR (Fig. 3B).

To confirm the inhibitory effects of WFA on EPCR shedding, TNF-α or interleukin (IL)-1β was used because previous reports showed that EPCR was shed by TNF-α or IL-1β in HUVECs (Menschikowski et al., 2009). In agreement with previous results, we found that EPCR shedding by TNF-α or interleukin (IL)-1β increased and WFA also suppressed TNF-α or IL-1β-mediated EPCR shedding in HUVECs (Fig. 3C and D). Because TNF-α and IL-1β have been shown to be important mediators during endotoxemia (Kremer et al., 1996; Michie et al., 1988), current finding showing that WFA inhibited TNF-α or IL-1β-mediated EPCR shedding could support our recent finding that WFA have anti-inflammatory responses in human endothelial cells and in CLP-induced septic mice (Lee et al., 2012).

3.2. Effects of WFA on PMA-stimulated expression and activity of TACE

A previous study reported that PMA-stimulated EPCR shedding is mediated by tumor necrosis factor-α converting enzyme/ADAM17 (TACE) (Qu et al., 2007). In order to determine whether WFA could inhibit stimulation of TACE expression and activity, endothelial cells were pretreated with increasing concentrations of WFA for 6 h, followed by stimulation with 1 μM PMA for 1 h. Data showed that WFA inhibited TACE expression (Fig. 4A) and activity (Fig. 4B) induced by PMA in endothelial cells.

3.3. Effect of WFA on CLP-induced EPCR shedding

To confirm the inhibitory effects of WFA on EPCR shedding in vivo, we used a CLP mouse model, because this model more closely resembles human sepsis (Buras et al., 2005; Yang et al., 2004). In CLP-induced septic mice, immunohistochemical analysis showed that lower expression of cellular EPCR than that of the control in aorta of mouse (Fig. 5A). Administration of WFA at a single dose (1.882 μg, 12 h after CLP) did not result in prevention of CLP-induced EPCR shedding (Fig. 5B); therefore, it was administered twice (1.882 μg per mouse, once 12 h, then 50 h after CLP), resulting in a decrease in EPCR shedding (Fig. 5C). Assuming an average body weight of 20 g and an average blood volume of 2 ml if no plasma proteins bind to WFA, the amounts of WFA produced a concentration of approximately 2 μM in peripheral blood. This marked benefit achieved by administration of WFA suggested that inhibition of EPCR shedding provides a therapeutic strategy for management of severe vascular diseases.

3.4. Effects of WFA on PMA-stimulated phosphorylation of p38MAPK, ERK1/2, and JNK

Previous studies have reported the involvement of p38MAPK, ERK1/2, and JNK in cytokine-induced EPCR shedding and phosphorylation of p38MAPK, ERK1/2, and JNK was known to be increased by stimulation with PMA (Han et al., 2010; Leng et al., 2004; Menschikowski et al., 2009). Therefore, in order to determine the molecular mechanisms of suppression of PMA-induced EPCR shedding by WFA, the effects of WFA on PMA-stimulated phosphorylation of p38MAPK, ERK1/2, and JNK were tested. Treatment with WFA (1–2 μM) resulted in reduction of PMA-stimulated phosphorylation of p38, ERK1/2, and JNK (Fig. 6A). To confirm the involvement of MAPK on the EPCR shedding, a panel of known pharmacological inhibitors, SB-203580 (an inhibitor of p38MAPK), PD-98059 (an inhibitor of ERK 1/2), and SP-600125 (an inhibitor of JNK), respectively, was cross-investigated by using HUVECs. Distinct attenuation of sEPCR release in HUVECs was observed after treatment with PD-98059, SB-203580, and SP-600125 (Fig. 6D). These results indicate that WFA inhibited PMA-stimulated phosphorylation of p38, ERK1/2, and JNK. Therefore, WFA might inhibit the EPCR shedding by suppressing PMA-stimulated activation of MAP kinases including p38, ERK and JNK directly or indirectly.

Metalloproteinase-mediated ectodomain shedding has been reported for many cellular receptors (Mooss and Lambert, 2002). TACE (ADAM17) is an important member of the ADAM (a disintegrin and metalloproteinase) family (Blobel, 2005). TACE and closely related...
Matrix metalloproteinases work together as sheddases to cleave hundreds of diverse transmembrane substrates, including TNF-α (Black et al., 1997), transforming growth factor-α, β-catenin (Lee et al., 2003), β-amyloid precursor protein (Buxbaum et al., 1998), and growth hormone receptor (Zhang et al., 2000). Unfortunately, little is known regarding which inhibitors modulate the activity or expression of sheddase and how such a broad palette of proteolytic activity integrates to modulate behaviors. Furthermore, therapeutics have targeted sheddases and their substrates, yet many of these inhibitors have failed in clinical trials (Fingleton, 2008). Therefore, a need exists for finding new inhibitors of TACE-mediated degradation, which integrates multiple layers of signaling networks to coordinately influence cell behavior in various vascular inflammatory diseases.

There is increasing evidence that diminished EPCR expression and function can contribute to chronic inflammation and autoimmune disease. For example, individuals with active inflammatory bowel disease exhibit depleted EPCR expression and increased EPCR shedding on their colonic mucosal microvasculature, caused by local generation of TNF-α and IL-1β in the inflamed local environment (Scaldaferri et al., 2007). The deleterious effect of vascular EPCR depletion in vivo was highlighted by mice with dextran-sodium sulfate-induced colitis, who had lost the ability to effectively generate APC. Replacement of the missing APC by recombinant APC administration reduced disease activity, weight loss, and mucosal inflammation by inhibition of chemokine production and leukocyte adhesion to the colonic microvascular endothelium (Scaldaferri et al., 2007). Furthermore, the levels of plasma...
sEPCR were elevated in patients with inflammatory diseases (Kurosawa et al., 1998; Sesin et al., 2005; Ware et al., 2004). These previous findings may support the fact that increased shedding of EPCR is associated with the hypercoagulopathy frequently observed in inflammatory conditions (Esmo, 2005). EPCR can contribute to anti-inflammatory mechanisms that involve protease activated receptor-1 on the surface of endothelial cells because sEPCR effectively inhibits the anti-inflammatory and anti-apoptotic functions of APC in human endothelial cells (Bae et al., 2007). In addition, given the established link between chronic inflammation and vascular disease, the impairment of the EPCR-dependent APC cytoprotective function may also contribute to the deleterious effect of anti-EPCR auto-antibodies in vascular disease. In this study, we demonstrated the inhibitory effects of WFA on EPCR shedding by PMA-, TNF-α, IL-1β, and CLP-mediated EPCR shedding. Therefore, WFA could be another potential candidate for treatment of vascular inflammatory diseases.

Phosphorylation levels of p38MAPK, ERK1/2, and JNK are increased by stimulation with PMA (Han et al., 2010; Leng et al., 2004; Menschikowski et al., 2009), and activation of TACE occurs upon activation of ERK or p38 MAPK (Huovila et al., 2005; Murphy, 2008). Therefore, PMA-activated TACE is the downstream signaling molecules of MAP kinases. In order to define the processes responsible for inhibition of PMA-stimulated shedding of EPCR and expression of TACE by WFA, we investigated the involvement of MAPK signaling pathways under PMA-stimulated conditions. MAPKs comprise a family of highly conserved serine/threonine protein kinases implicated as having key regulatory roles in mediating inflammation (Thalhammer et al., 2008). Three major classes of MAPKs are represented by ERK 1/2 and the 2 stress-activated protein kinase families JNK and p38 MAPK. As shown in Figs. 4 and 6, WFA remarkably inhibited PMA-stimulated expression of TACE and phosphorylation of p38 MAPK, ERK1/2, and JNK to show WFA as an inhibitor of PMA-stimulated EPCR shedding. Therefore, WFA inhibited PMA-stimulated shedding of EPCR via indirectly inhibiting the expression and activity of TACE and MAPK.

We recently reported an anti-inflammatory property of WFA against high mobility group box 1 protein (HMGB1) and the associated signaling pathways-induced inflammatory response using HUVECs and mice (Lee et al., 2012). We reported that WFA inhibited lipopolysaccharide (LPS)-induced HMGB1 release and HMGB1-mediated barrier disruption, expression of cell adhesion molecules (CAMs) and adhesion/transendothelial migration of leukocytes to human endothelial cells. WFA also suppressed acetic acid-induced hyperpermeability and carboxymethylcellulase-induced leukocytes migration in vivo. Further studies revealed that WFA suppressed the production of IL-6, TNF-α and activation of nuclear factor-κB (NF-κB) by HMGB1 (Lee et al., 2012).

Collectively, the results of this study show that WFA induced potent inhibition of PMA, TNF-α, IL-1β and CLP-induced EPCR shedding and it suppressed the expression and activation of TACE. Noting that EPCR shedding is an important step involved in the pathophysiological pathway of vascular inflammatory diseases; the hypothesis that WFA could be used as a candidate therapeutic for treatment of vascular inflammatory diseases has strengthened by the finding of our previous study (Lee et al., 2012) and the current findings. Even though the use of WFA for therapeutic purposes could have non-specific effects, the results of this study provide novel information on the role of WFA on EPCR shedding. Therefore, our findings suggest that WFA be a potential candidate for treatment against severe vascular inflammatory diseases, such as sepsis and septic shock.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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