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Inhibitory effects of lysozyme on endothelial protein C receptor shedding *in vitro* and *in vivo*

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Lysozyme protects us from the ever-present danger of bacterial infection and binds to bacterial lipopolysaccharide (LPS) with high affinity. Beyond its role in the activation of protein C, the endothelial cell protein C receptor (EPCR) plays an important role in the cytoprotective pathway. EPCR can be shed from the cell surface, which is mediated by tumor necrosis factor-α converting enzyme (TACE). However, little is known about the effects of lysozyme on EPCR shedding. We investigated this issue by monitoring the effects of lysozyme on phorbol-12-myristate 13-acetate (PMA)-, tumor necrosis factor (TNF)-α-, interleukin (IL)-1β-, and cecal ligation and puncture (CLP)-mediated EPCR shedding and underlying mechanism. Data demonstrate that lysozyme induced potent inhibition of PMA-, TNF-α-, IL-1β-, and CLP-induced EPCR shedding. Lysozyme also inhibited the expression and activity of PMA-induced TACE in endothelial cells. These results demonstrate the potential of lysozyme as an anti-EPCR shedding reagent against PMA-mediated and CLP-mediated EPCR shedding. [BMB Reports 2015; 48(11): 624-629]

INTRODUCTION

Endothelial protein C receptor (EPCR) is one of the type I trans-membrane protein with homology to CD1/MHC class I trans-membrane proteins which are engaged in vascular inflammation (1). As a receptor to protein C (PC) and activated protein C (APC) with the high binding affinity to them, EPCR has a anticoagulant and anti-inflammatory functions (2, 3). The anti-

coagulant pathway of APC is induced by the activated blood coagulation factors Va and VIIIa with the involvement of protein S, high-density lipoproteins, phosphatidylserine, cardiolipin, and glucosylceramide (4). The cytoprotective functions of APC such as anti-inflammatory, anti-apotosis, and vascular barrier protective, is mediated by EPCR and the protease-activated receptor-1 (PAR-1) (4).

By EPCR shedding processes, a soluble form of EPCR (sEPCR) can be generated *in vitro* through proteolytic cleavage by metalloprotease activity, which is inducible by several inflammatory mediators (5, 6). Circulating EPCR in plasma could bind to both APC and PC, but could not enhance the activation of PC to APC (7). And, sEPCR suppresses APC's anticoagulant activity via the formation of a complex that does not bind to phospholipid membranes (8). Owing to the shedding of membrane EPCR, sEPCR can be detected in plasma at a concentration of approximately 100 ng/mL, and high levels of sEPCR have been reported in systemic inflammatory diseases (9). Previous *in vitro* reports showed a significant increase sEPCR in endothelial cells by a variety of vascular inflammatory inducers such as interleukin (IL)-1β, hydrogen peroxide, and phorbol myristate acetate (PMA), and thrombin, and EPCR shedding is potentiated by nocodazole (5, 6, 10). And, the activation of p38 MAPK, ERK1/2, and JNK was mediated by PMA (11-13), and activation of tumor necrosis factor-α converting enzyme (TACE) occurs upon activation of ERK or p38 (14, 15).

Lysozymes thwart bacterial growth and are found in relatively high concentration in blood, saliva, tears, and milk (16). Lysozyme is a small protein that protects us from the ever-present danger of bacterial infection by attacking the cell walls of bacteria (17, 18). Cell wall of bacteria, composed of carbohydrate chains, braces their delicate membrane against the cell's high osmotic pressure and lysozyme breaks these carbohydrate chains, which leads to the rupturing of the bacteria under their own internal pressure (17, 18).

Lysozyme is one of the powerful first-line defenses against bacterial infection, where microorganisms are most likely to enter the body (17, 18). However, to date, the effects of lysozyme on EPCR shedding have not yet been studied. Thus, noting that lysozyme has a pleiotropic role in bacterial defense, and that

shedding of EPCR is accompanied with the disruption of vascular integrity and the main pathophysiology of sepsis (9, 19), we hypothesized that lysozyme might inhibit the shedding of EPCR. Now, we report evidence to support our hypothesis.

RESULTS AND DISCUSSION

Effect of lysozyme on PMA-, TNF-α-, or IL-1β-induced EPCR shedding

Previous studies have demonstrated the PMA stimulation of EPCR shedding from human umbilical vein endothelial cells (HUVECs) (20, 21). In agreement with those studies, our results show that 1 μm PMA fully stimulated EPCR shedding from HUVECs (Fig. 1A), and induced a decrease in membrane EPCR on HUVECs (Fig. 1B). To investigate the effect of lysozyme on PMA-mediated EPCR shedding, HUVECs were pretreated with increasing concentrations of lysozyme for 6 h, followed by stimulation with 1 μm PMA for 1 h. Results showing that lysozyme inhibited EPCR shedding induced by PMA in HUVECs, with an op-

timal effect at 20-200 nm are presented in Fig. 1A and B. However, lysozyme alone had no effect on EPCR shedding (Fig. 1A, B). Therefore, lysozyme alone did not affect the expression of membrane-bound EPCR. To confirm the inhibitory effects of lysozyme on EPCR shedding, TNF-α or IL-1β was used because previous reports have shown that EPCR shedding was induced by TNF-α or IL-1β in HUVECs (11). We found that EPCR shedding induced by TNF-α or IL-1β increased and that lysozyme suppressed TNF-α-mediated (Fig. 1C) or IL-1β-mediated EPCR shedding (Fig. 1D) in HUVECs. Because endothelial cells exhibit differences in gene and protein expression according to their vascular bed of origin, we used human pulmonary artery endothelial cells to confirm the anti-EPCR shedding effects of lysozyme against PMA-, TNF-α-, or IL-1β-induced EPCR shedding (data not shown). Because TNF-α and IL-1β have been shown to be important mediators of endotoxemia (22, 23), the current findings showing that lysozyme inhibited TNF-α-mediated or IL-1β-mediated EPCR shedding could support the notion that lysozyme has anti-inflammatory effects in human endothelial cells.

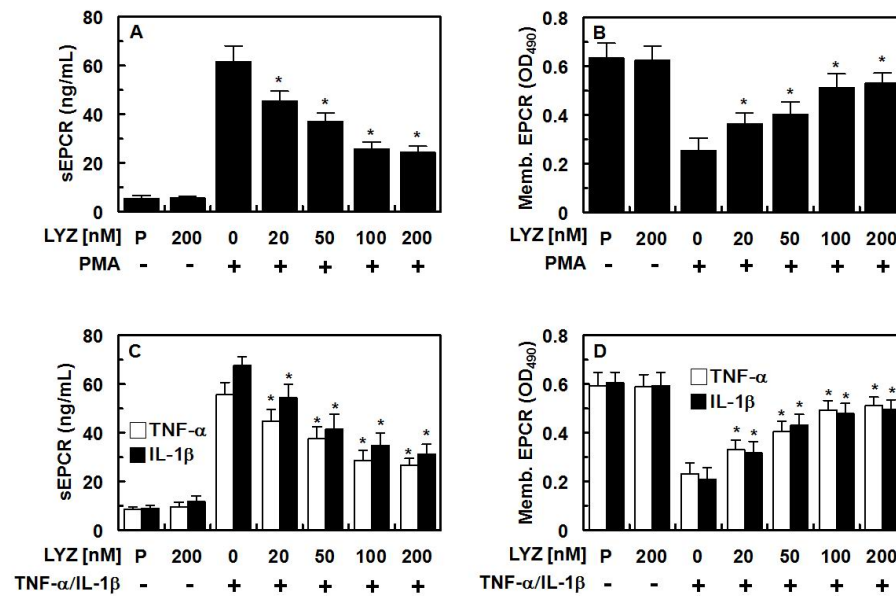**Fig. 1.** Effect of lysozyme on PMA-, TNF-α-, and IL-1β-induced EPCR shedding. The effects of various concentrations of lysozyme on PMA (1 μm)-induced EPCR shedding were monitored by the measurement of sEPCR (A) or membrane EPCR on HUVECs (B) and after HUVEC incubation with TNF-α (C, 25 ng/mL for 1 h, white bar) or IL-1β (D, 25 ng/mL for 1 h, black bar). "P" in each X-axis means vehicle (PBS) only. Results are expressed as the mean ± SEM of three separate experiments. *P < 0.05 vs. PMA alone (A, B), TNF-α (C, D), or IL-1β alone (C, D).
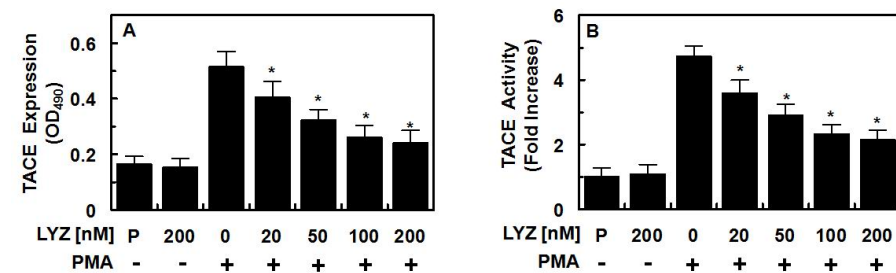

Fig. 2. Effect of lysozyme on PMA-induced expression and activity of TACE. The effects of various concentrations of lysozyme on PMA (1 μm, 1 h)-induced expression of TACE were monitored by measurement of TACE ELISA (A), or TACE activity (B). "P" in each X-axis indicates vehicle (PBS) only. All results are expressed as the mean ± SEM of three separate experiments. *P < 0.05 vs. PMA alone.

Effects of lysozyme on PMA-stimulated expression and activity of TACE

Previously, TACE mediates PMA-stimulated EPCR shedding (21). To verify if lysozyme can suppress the expression and activity of TACE by PMA, HUVECs were stimulated with PMA after treated with lysozyme. Data showed that lysozyme inhibited TACE expression by PMA (Fig. 2A). PMA-induced TACE activity was also inhibited by lysozyme (Fig. 2B).

Effect of lysozyme on CLP-induced EPCR shedding, protein C concentration, and thrombin generation

To confirm the effects of lysozyme on EPCR shedding in mice,

we used a CLP model because this model could mimic severe human vascular inflammatory diseases, sepsis (24-26). To determine the trend in mortality caused by CLP, groups of septic and sham-operated mice were monitored for survival for 132 h (>five days). The survival rate of mice subjected to CLP was 20% at 80 h and 0% at 100 h post operation (Fig. 3A). In CLP-operated mice, histological data showed the decreased expresson of membrane EPCR compared to controls (Fig. 3B). Injection of lysozyme at a single dose (2.86 μg/mouse, 12 h after CLP) could not inhibit EPCR shedding by CLP (Fig. 3C). Thus, lysozyme was injected two times (once at 12 h, then at 50 h post CLP), resulting in a decrease in EPCR shedding (Fig.
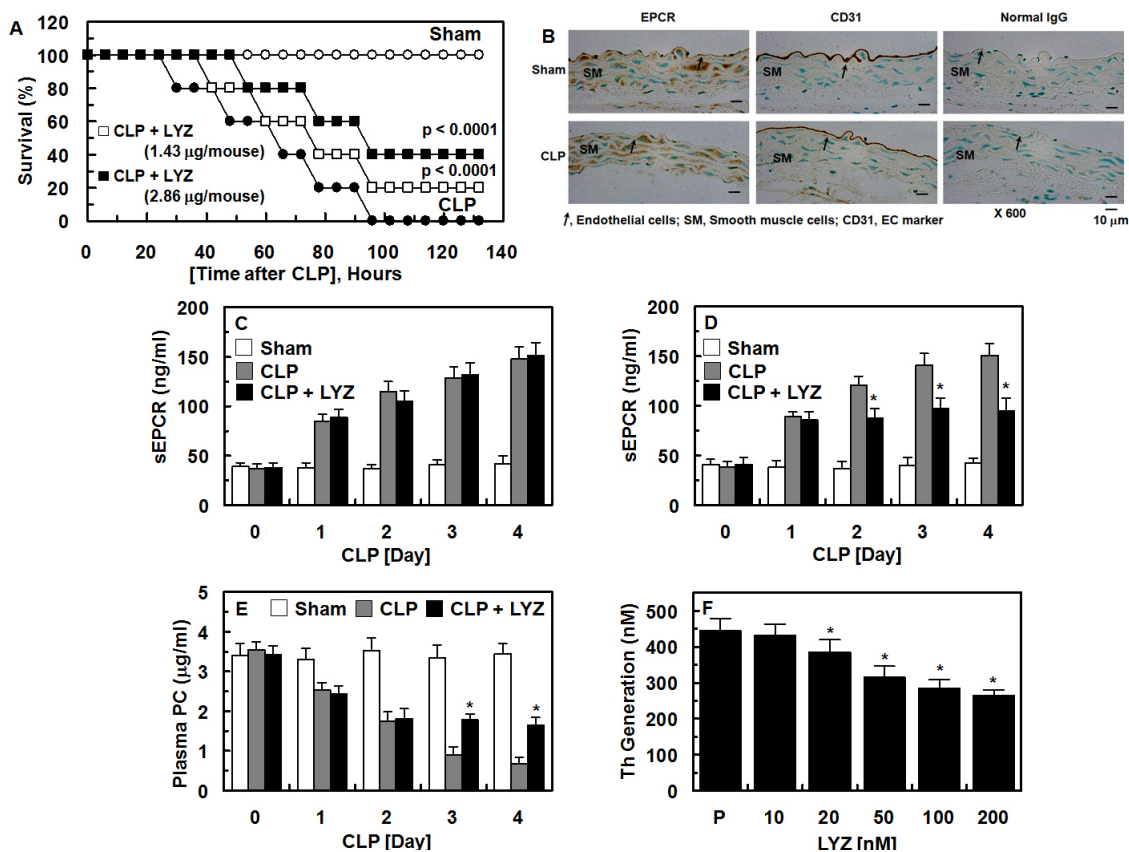

Fig. 3. Effect of lysozyme on CLP-induced mortality, EPCR shedding, PC concentrations, and thrombin generation. (A) Male C57BL/6 mice (n = 20) were administered intravenously with lysozyme (1.43 μg/mouse, □) or (2.86 μg/mouse, ■) at 12 h and 50 h after CLP. Animal survival was monitored every 6 h after CLP for 132 h. Control CLP mice (●) and sham-operated mice (○) were administered sterile saline. A Kaplan-Meier survival analysis was used for the determination of overall survival rates vs. CLP treated mice. (B) Immunohistochemical staining of the descending thoracic aortic vessel for membrane EPCR from CLP-operated mice was compared with results from sham-operated mice. Results are representative of 3-6 staining processes from 3 independent experiments per condition. (C-E) Serum was obtained from sham-operated (white bar) or CLP-induced septic mice (gray bar) on the indicated day after CLP surgery (n = 5 each group) on different days for different mice. Lysozyme (black bar, 2.86 μg/mouse) was administered once (C, 12 h after CLP) or twice (D and E, once 12 h, then 50 h after CLP) on different days for different mice. EPCR shedding was then monitored by measurement of sEPCR (C, D) and plasma PC was determined by ELISA (E). (F) HUVEC monolayers were pre-incubated with FVa (100 pM) and FXa (1 nM) for 10 min with the indicated concentrations of lysozyme. Prothrombin was added to a final concentration of 1 μm and prothrombin activation was determined 30 min later, as described in the "Materials and Methods" section. "P" in each X-axis indicates vehicle (PBS) only. All results are expressed as the mean ± SEM of three separate experiments. *P < 0.05 vs. CLP alone (C-E) or vs. P (F).

3D). It is well known that average circulating blood volume for mice is 72 ml/kg (27). As the average weight of used mice in this study is 27 g, and the average blood volume is 72 mL/kg (2 ml/27 g), the amount of lysozyme (2.86 μg per mouse) injected yielded a maximum concentration of 100 nm in the peripheral blood, which was determined by ELISA. Based on these findings, we hypothesized that treatment with lysozyme could reduce the CLP-induced sepsis lethality. Administration of lysozyme (1.43 μg or 2.87 μg/mouse, once at 12 h, then at 50 h after CLP) resulted in an increase in the survival rate to 20-40% (P < 0.0001, Fig. 3A). This result suggests that the suppression of EPCR shedding provides a therapeutic strategy for the management of sepsis.

The main enzyme in the PC-mediated negative feedback mechanism is APC, which proteolytically cleaves FVa and FVIIIa to inhibit the generation of thrombin and subsequent fibrin formation (4, 28). PC is converted to APC by the interaction of thrombin and thrombomodulin (4, 28). Therefore, we tested the effects of lysozyme on PC concentration. The data show that plasma PC levels were significantly attenuated in CLP-operated mice and administration of lysozyme suppressed the CLP-induced decrease in PC levels (Fig. 3E). In addition, activation of the coagulation cascade appears to be an essen-

tial component in the development of multi-organ failure in sepsis (29).

Therefore, we tested the effects of lysozyme on the generation of thrombin in HUVECs. Previous report showed that HUVECs are able to support the activation of prothrombin by factor Xa (FXa) (30). Here, pre-incubation of HUVECs with FVa and FXa in the presence of $CaCl_2$ prior to addition of prothrombin resulted in production of thrombin (Fig. 3F). And, treatment with lysozyme inhibited the production of thrombin from prothrombin by dose-dependent manner (Fig. 3F). Lysozyme at 200 nm did not have antibacterial activity, which excludes the possibility that the inhibition of EPCR shedding, TACE activity, and thrombin generation by lysozyme is not due to the antibacterial activity of lysozyme (data not shown).

Effects of lysozyme on PMA-stimulated phosphorylation of p38 MAPK, ERK1/2, and JNK

Previous studies have reported the involvement of p38 MAPK, ERK1/2, and JNK in cytokine-induced EPCR shedding, and increased phosphorylation of p38 MAPK, ERK1/2, and JNK has been known to occur via PMA stimulation (11-13). Therefore, in order to determine the molecular mechanisms underlying the suppression of PMA-induced EPCR shedding by lysozyme, the
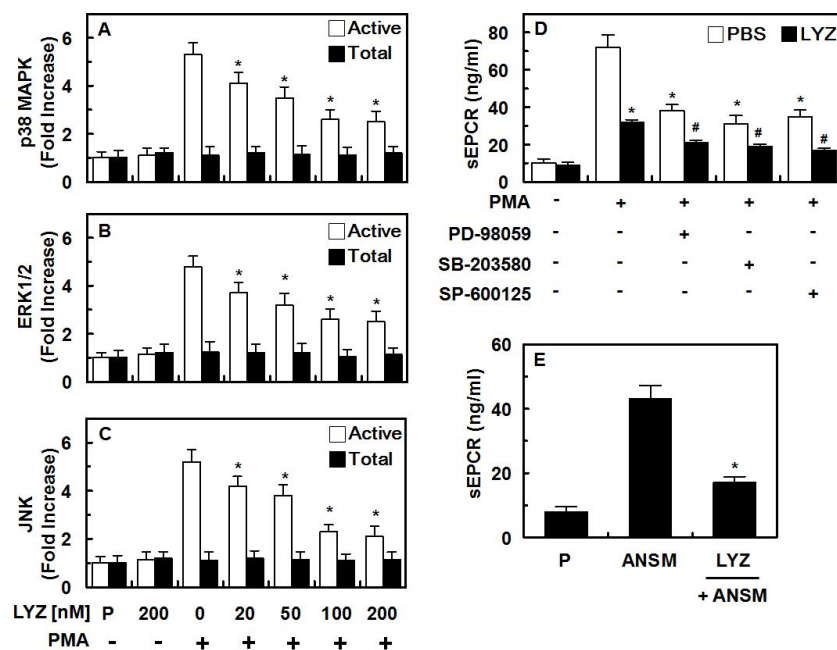

Fig. 4. Effect of lysozyme on PMA-induced phosphorylation of p38 MAPK, ERK1/2, and JNK. (A-C) PMA (1 μm, 1h)-mediated phosphorylation of phospho-p38 MAPK (A; white bar), total p38 MAPK (A; black bar), phospho-ERK1/2 (B; white bar) or total ERK1/2 (B; black bar) and phospho-JNK (C; white bar) or total JNK (C; black bar) was analyzed after the treatment of cells with lysozyme. Results are expressed as fold increases over control values. (D) Cells were pre-incubated with 50 μm PD-98059, 10 μm SB-203580, or 20 μm SP-600125 as indicated for 30 min with or without lysozyme at 100 nM each and thereafter exposed to PMA at a final concentration of 1 μm for an additional 1 h. EPCR shedding was monitored by measurement of sEPCR ELISA. (E) The effects of lysozyme at 100 nM each on Anisomycin (ANSM, 5 μm, 4 h)-induced EPCR shedding were monitored by the measurement of sEPCR. "P" in each X-axis indicates vehicle (PBS) only. All results are expressed as the mean ± SEM of three separate experiments. *P < 0.05 vs. PMA (A-D) alone, ANSM (H) alone, or #P < 0.05 vs. white bar (D).

effects of lysozyme on the PMA-stimulated phosphorylation of p38 MAPK, ERK1/2, and JNK were tested. As shown in Fig. 4A-C, treatment with lysozyme resulted in a reduction in PMA-stimulated phosphorylation of p38 MAPK (Fig. 4A), ERK1/2 (Fig. 4B), and JNK (Fig. 4C). A panel of pharmacological inhibitors of MAP kinases was used to confirm the involvement of MAPK in EPCR shedding. As shown in Fig. 4D, a distinct attenuation of sEPCR release in HUVECs was observed after treatment with PD-98059 (a pharmacological inhibitor of ERK 1/2 activity), SB-203580 (an inhibitor of p38 MAPK), and SP-600125 (an inhibitor of JNK). We also found synergistic anti-EPCR shedding effects of each MAP kinase inhibitor with lysozyme on the PMA-mediated EPCR shedding (Fig. 4D). Furthermore, MAP kinase signaling pathways such as JNK and p38 are directly targeted by lysozyme in response to stimulation of EPCR shedding because anisomycin (activator of JNK and p38 MAPK)-mediated EPCR shedding is reduced by lysozyme (Fig. 4E).

As the increased phosphorylation of p38 MAPK, ERK1/2, and JNK is known to occur via PMA stimulation (11-13) and the activation of TACE occurs upon activation of ERK or p38 MAPK (14, 15), we aimed to determine the processes responsible for the inhibition of PMA-stimulated EPCR shedding and expression of TACE by lysozyme. To do this we investigated the involvement of MAPK signaling pathways under PMA-stimulated conditions. MAPKs comprise a family of highly conserved serine/threonine protein kinases that are believed to play key roles in the mediation of inflammation (31). Three major classes of MAPKs are represented by ERK 1/2 and the stress-activated protein kinase families, JNK and p38 MAPK. As shown in Figs. 2 and 4, lysozyme inhibited the responses of PMA-stimulated expression of TACE and phosphorylation of p38 MAPK, ERK1/2, and JNK. Therefore, lysozyme inhibited EPCR shedding via the inhibition of the PMA-stimulated expression of TACE and the activation of MAPKs.

Collectively, the results of this study show that lysozyme potently inhibited PMA-, TNF-α-, IL-1β- and CLP-induced EPCR shedding and suppressed the expression and activity of TACE (Supplementary Fig. 1). Lysozyme also suppressed the CLP-induced PC decrease in mice and thrombin generation in HUVECs. In addition, lysozyme reduced PMA-stimulated phosphorylation of p38 MAPK, ERK 1/2, and JNK. TACE induced the shedding of a large number of cell membrane-bound proteins, and in the present study, EPCR shedding was also shown to be mediated by TACE, which was inhibited by lysozyme. Selective targeting and inhibition of sEPCR by lysozyme in the endothelium against several inducers such as PMA, TNF-α, IL-1β, and CLP, affords promise for severe vascular inflammatory diseases such as sepsis. However, pharmacological and pharmaceutical factors such as adsorption to the endothelium, distribution, metabolism, and excretion are major hurdles to overcome before lysozyme's therapeutic potential can be fully realized. Although the use of lysozyme for therapeutic purposes could have non-specific effects, the data presented in this study provide novel information on the role of lysozyme in EPCR shedding.

Therefore, our findings indicate the potential of lysozyme as a candidate for the treatment of severe vascular inflammatory diseases, such as sepsis and septic shock.

MATERIALS AND METHODS

Reagents
Lysozyme from chicken egg white (L7651), anisomycin, and IL-1β were purchased from Sigma-Aldrich (St. Louis, MO). sEPCR and TNF-α were purchased from Abnova (Taiwan). FVa, FXa, prothrombin, and thrombin were obtained from Haematologic Technologies (Essex Junction, VT).

Cell culture
Primary HUVECs were obtained from Cambrex Bio Science (Charles City, IA) and maintained as previously described (10, 26, 32). HUVECs from passage 3 or 4 were used in the subsequent experiments.

TACE activity assay
For the TACE activity assay, a commercially available TACE activity kit (Innozyme TACE activity assay kit, EMD Millipore, Billerica, MA) was used as described previously (10, 33).

Thrombin production on the surface of HUVECs
Measurement of thrombin production by HUVECs was quantified as previously described (34, 35).

Competitive ELISA for sEPCR and TACE
The concentrations of sEPCR and TACE were measured as described previously (10).

Statistical analysis
All experiments were performed independently at least three times. Values are expressed as mean $\pm$ standard error of the mean (SEM). The statistical significance of differences between test groups was evaluated by one-way analysis of variance (ANOVA) and Tukey's post-hoc test. SPSS for Windows, version 16.0 (SPSS, Chicago, IL) was used to perform statistical analysis, and statistical significance was accepted for P values < 0.05.

ACKNOWLEDGEMENTS

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