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SSeCKS/Gravin/AKAP12 Inhibits PKCζ-Mediated Reduction of ERK5 Transactivation to Prevent Endotoxin-Induced Vascular dysfunction

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Abstract

SSeCKS/Gravin/AKAP12 is a protein kinase C (PKC) substrate that inhibits the activity of PKC through binding with it. SSeCKS is expressed in vascular endothelial cells (ECs). The atypical PKC isoform ζ (PKCζ) is a pathologic mediator of endothelial dysfunction. However, the functional significance of SSeCKS/PKCζ dimerization in the vascular endothelium remains poorly understood. Given this background, we investigated the effects of SSeCKS on endothelial dysfunction and elucidated the possible mechanism involved. Vascular endothelial dysfunction and inflammatory changes were induced by treatment with bacterial endotoxin lipopolysaccharide (LPS, a vascular endothelial toxicity inducer). LPS can increase the level of SSeCKS. However, we also found that depletion of SSeCKS aggravated the LPS-induced vascular endothelial dysfunction, upregulated pro-inflammatory proteins and phosphorylation level of PKCζ, increased ROS formation, decreased extracellular-signal-regulated kinase 5 (ERK5) transcriptional activity, and reduced eNOS expression. Further examination revealed that depletion of SSeCKS increased PKCζ/ERK5 dimerization. These findings provide preliminary evidence that the expression of SSeCKS induced by LPS, as a negative feedback mechanism, has the potential to improve endothelium-dependent relaxation in vascular disease conditions by inhibiting PKCζ-mediated reduction of ERK5 transactivation.

Keywords SSeCKS · PKCζ · Lipopolysaccharide · Vascular toxicity · ERK5

Abbreviations

PKCζ	Protein kinases C isoformsζ
ECs	Vascular endothelial cells
LPS	Lipopolysaccharide
ERK5	Extracellular-signal-regulated kinase 5
ACh	Acetylcholine

Introduction

SSeCKS/Gravin/AKAP12, a major protein kinase C (PKC) substrate [1], is a scaffold protein for PKC, which controls actin cytoskeleton reorganization and has been identified to be expressed in aortic endothelial cells (ECs) [2, 3]. SSeCKS is also a response protein of lipopolysaccharide (LPS) [4, 5]. Meanwhile, LPS can induce cytotoxicity in ECs [6]. However, the role of SSeCKS expression, which is induced by LPS in vascular endothelial dysfunction, is complicated and remains to be completely elucidated. Previous studies have shown that SSeCKS can inhibit PKC activity through direct scaffolding of PKC isozymes [7]. PKCζ emerged as a pathologic mediator of endothelial dysfunction [8]. However, the role of SSeCKS/PKCζ dimerization in endothelial dysfunction and the molecular mechanisms have not been fully explained.

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ECs provide a functional barrier and modulate several signals involved in vasomotion [9–11]. Previous findings indicate that LPS is a major causal factor in the development of vascular endothelial dysfunction [12, 13]. The reaction of ECs to LPS results in downregulation of eNOS expression [14, 15], upregulation of inflammatory protein [16, 17], and impairment of endothelium-dependent vasodilation [11, 18]. The essential cellular event for the initiation of inflammatory processes associated with vascular dysfunction is monocyte migration to the inflammation site and its subsequent adhesion to ECs [19]. These processes are controlled by interaction between numbers of vascular cell and adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1). As described previously, LPS can activate the nuclear factor κB (NF-κB) pathway to elicit the expression of a series of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 [20, 21]. In addition, pro-inflammatory cytokine induces PKCζ activation, leading to inflammation via increasing PKCζ/ extracellular-signal-regulated kinase 5 (ERK5) dimerization and inhibiting ERK5 transcriptional activity, which downregulates eNOS and KLF2 expression [22]. Meanwhile, SSeCKS inhibits PKC activity through its scaffolding domains [2, 7]. However, how SSeCKS regulates PKCζ/ERK5 complex formation or whether it is involved in EC inflammation remains to be elucidated.

ERK5 is a member of the MAPK family [23, 24]. It is ubiquitously expressed in the EC and plays an crucial role in EC homeostasis [25, 26]. ERK5 is required for the activation of KLF2, which is responsible for negatively regulating EC inflammation, inducing eNOS expression, and maintaining vascular quiescence [27–29]. ERK5 activation also suppresses ROS generation in EC [30]. One study demonstrated that PKCζ binds directly to ERK5 through its scaffolding domains [22]. And, the PKCζ/ERK5 dimerization decreases eNOS expression and contributes to endothelial dysfunction [22].

In the present study, we focus on the following goals (i) to determine the role of SSeCKS in endothelial dysfunction and (ii) to investigate the molecular mechanisms of how PKCζ/ERK5 dimerization is enhanced by depleting SSeCKS expression in vascular endothelial dysfunction.

Methods

Animal

Twelve-week-old Male C57BL/6 mice were maintained on a 12-h light/dark cycle at a constant room temperature (22 °C ± 1 °C). The current study was performed in adherence to the National Institutes of Health guidelines for the use of experimental animals, and all animal protocols were approved by the Committee for Ethical Use of Experimental Animals of the Fourth Military Medical University.

Vascular Reactivity

The animals were anesthetized by the intraperitoneal administration of 20% urethane. Aorta from the heart to the iliac bifurcation was removed and placed in ice-cold Krebs buffer consisting of (mM) NaCl, 118; KCl, 4.8; $CaCl_2{\cdot}2H_2O$, 2.5; $MgCl_2{\cdot}6H_2O$, 2.5; $NaH_2PO_4{\cdot}2H_2O$, 1.2; $NaHCO_3$, 8.5; and glucose·H_2O, 11. The aorta was cleared of fat, as well as connective tissue, and cut into rings 2 mm long. The rings were mounted onto hooks, suspended in organ chambers filled with Krebs buffer, aerated with 95% O_2/5% CO_2 at 37 °C, and connected to pressure transducers (WPI, Sarasota, FL) to record the changes via a Mac-Lab recording system. After 30 min of equilibration at an optimal preload of 9.8 mN, the rings were stimulated with 1 mM of phenylephrine (PE, Sigma-Aldrich, St. Louis, MO, USA) and 1 mM of acetylcholine (ACh, Sigma-Aldrich, St. Louis, MO, USA) (both from Sigma-Aldrich, St. Louis, MO), and rings with > 50% relaxation were considered endothelium-intact.

Plasmid

The Mouse SSeCKS gene was cloned into pCMV6-Entry vector (Origene). The pCMV6-Entry vector is C-terminally tagged with Myc and Flag. Mutations in the SSeCKS gene(the △553–900 mutant) were generated in pCMV6-Entry vectors with the full-length wild-type SSeCKS gene. Adenoviruses that drive expressions of the form of PKCζ and ERK5 (Myc-DDK-tagged) were purchased from cell biolabs. Mutagenesis of ERK5 was performed using the QuikChange site-directed mutagenesis kit (Agilent). Adenovirus vector containing β-galactosidase (Ad-LacZ) was used as a control virus. shRNA Lentiviral Particles specific for mouse SSeCKS, PKCζ, ERK5, and scrambled control were provided by Santa Cruz Biotechnology Inc (CA). Control shRNA lentiviral particles (Santa Cruz Biotechnology) were used to confirm the selectivity of lentiviruses.

Cell Culture and Transfection

Human umbilical vein ECs (HUVECs) and HEK293T cells were maintained in RPMI 1640 medium (HyClone, UT, USA) supplemented with heat-inactivated fetal bovine serum (10%), 2 mM of L-glutamine, 100 U/mL of penicillin, and 100 g/mL of streptomycin. Then, the cells were incubated at 37 °C in 5% CO_2 and 95% air. For transient transfections, cells were seeded on six-well plates and allowed to grow to 70–80% (cells per plate) confluence at the time of transfection. In general, Myc-DDK–PKCζ, Myc-DDK–ERK5, and Myc-DDK–SSeCKS expression vectors were used,

or otherwise, as indicated. HEK 293T cells were transfected using LipofectAmine 2000 (Invitrogen). Forty-eight hours after transfection, cells were used for experiments as described. NO levels in culture medium were assayed by NO detection kit (Nanjing Jiancheng Bioengineering Institute, China).

Lentiviral RNA Interference on Mouse Aortae

Lentivirus particles of SSECKS shRNAs and control shRNAs were prepared as previous described. Mouse aortic rings were transfected with lentivirus (106 pfu) in the presence of 8 μg/mL polybrene (Sigma) for 4 h in FBS-free DMEM, and then transferred to DMEM with 10% FBS for both functional and molecular examination.

Immunoprecipitation and Western Blot Analysis

Immunoprecipitation was performed using a Dyna-Beads Protein G immunoprecipitation kit (Invitrogen). Cells were harvested and extracted for protein using IP lysis buffer (Thermo Scientific). Two hundred micrograms of whole-cell protein were precipitated at 4 °C with anti-Myc-DDK (dilution ratio 1:200,Origene) and anti-ERK5 (dilution ratio 1:1000; Cell signaling). The immunoprecipitates were eluted and analyzed by western blotting.

As described previously [31], equal amounts of proteins (30 μg) from the cultures or the aortas were separated and electrotransferred onto the NC membranes (Invitrogen, Carlsbad, USA) that were probed with anti-ERK5 (dilution ratio 1:1000; Cell signaling), anti-PKCζ (dilution ratio 1:1000; Cell signaling), anti-phospho-PKCζ (dilution ratio 1:1000; Cell signaling), Anti-SSeCKS (1:250; Santa), and Anti-GAPDH (dilution ratio 1:10000; Sigma-Aldrich, St. Louis, MO) antibodies diluted in blocking buffer. Each primary antibody was incubated overnight at 4 °C. After three washes with Tris-buffered saline and tween 20 for approximately 15 min, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit/anti-mouse immunoglobulin G for the primary antibodies), and the bands were visualized using an ECL system (PerkinElmer). The data were pooled from three independent experiments.

Immunocytochemistry

The cultured ECs were fixed with ice-cold 4% paraformaldehyde in PBS (pH 7.4) for 30 min, blocked with 5% BSA in PBS for 2 h, incubated overnight with primary anti- SSECKS (1:50) at 4 °C, and then incubated with Cy3-conjugated goat anti-rabbit IgG (Sigma) diluted to 1:100 in blocking solution. Coverslips were mounted onto slides with 50% glycerin. The stained samples were photographed and were analyzed using an Olympus Fluoview FV100 (Olympus, Japan).

Flow Stimulation

Confluent HUVECs cultured in 100-mm dishes were exposed to laminar flow as previously described [32] in a cone and plate viscometer placed in a cell incubator with 5% CO_2 and at 37 °C for 24 h (shear stress = 12 dyn/cm^2).

ROS Measurement

ROS production was measured by dihydroethidium (DHE, Sigma, excitation: 535 nm; emission: 610 nm) fluorescence [33]. The aortic segments were treated with 0.5 μM DHE solution to stain for O_2^- for 30 min at 37 °C and washed with room temperature saline three times for 5 min each. The specimens were then cut open and imaged by a confocal laser scanning microscope (Olympus, Tokyo, FV1000). HUVECs were washed with serum-free RPMI culture medium and incubated with 5 μmol/l dihydroethidium (DHE,Beyotime) at 37 °C for 30 min. Fluorescent images were captured by confocal system.

In Vitro Kinase Assay of ERK5

ERK5 kinase activity was measured by autophosphorylation and myelin basic phosphorylation. Comparison of ERK5 activity measured by ^{32}P incorporation into soluble myelin basic protein versus autophosphorylation of ERK5 showed a good correlation between the two techniques. Because autophosphorylation of immunoprecipitated ERK5 was more robust than myelin basic protein phosphorylation, we report only results from autophosphorylation assays. Cells were harvested in lysis buffer at 4 °C, then flash-frozen on a dry ice/ethanol bath. After allowing the cells to thaw, cells were scraped off the dish and centrifuged at 14,000 × g (4 °C for 30 min), and protein concentrations were determined. ERK5 was immunoprecipitated by incubating 400 μg of protein from each sample with 3 μl of the rabbit polyclonal anti-BMK1 antibody for 3 h and adding 40 μl of a 1:1 slurry of protein A-Sepharose (Pharmacia) beads to the extract/antibody mixture and incubation for 1 h at 4 °C. The beads were washed two times with 1 ml of lysis buffer, two times with 1 ml of LiCl wash buffer (500 mM LiCl, 100 mM Tris–Cl, pH 7.6, 0.1% Triton X-100, 1 mM DTT), and two times in 1 ml of modified Buffer A (20 mM HEPES, pH 7.2, 2 mM EGTA, 10 mM $MgCl_2$, 1 mM DTT, 0.1%Triton X-100). ERK5 kinase activity of the immunoprecipitate was measured at 30 °C for 20 min in a reaction mixture (40 μl) containing 15 μM ATP, 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 3 μCi of [γ - ^{32}P] ATP. The reaction was terminated by adding 8 μl of 6 × electrophoresis sample buffer and boiling for 5 min. ERK5 autophosphorylation was determined by densitometry

of bands at the correct molecular weights analyzed using a microplate absorbance reader Biorad680 (Biorad, CA, USA).

Statistical Analysis

Data are expressed as mean ± SEM. Statistical comparisons were performed using *t*-test, and the differences between the multiple groups were assessed using one-way ANOVA. $p < 0.05$ was considered statistically significant.

Results

SSeCKS Attenuates Superoxides, Inhibits Adhesion Molecule Expression, Regulates PKCζ Activity, and Enhances eNOS Expression in HUVECs

The O_2^- levels of HUVECs were measured by DHE fluorescent signals [34]. In addition, we used shRNA to reduce SSeCKS expression and inhibit its activity (Supplementary Fig. 1). LPS increased O_2^- level, but this effect was aggravated by SSeCKS shRNA ($p < 0.05$, Fig. 1a, b).

We also observed that LPS significantly increased the level of SSeCKS [5]. In addition, we examined adhesion molecule expression and NF-kB activation induced by LPS [35, 36]. We found that LPS increased both NF-kB and VCAM-1 expression, which were significantly enhanced by SSeCKS shRNA (Fig. 1c, d; Supplementary Figs. 1, 5). All values are fold change over control shRNA (n = 5). These data suggest the crucial role of SSeCKS in regulating ECs inflammation.

We investigated the influence of SSeCKS depletion on the activation of PKCζ in HUVECs exposed to LPS. The phosphorylation levels of PKCζ increased substantially when HUVECs were treated with LPS for 6 h (Fig. 1c, e). Deletion of SSeCKS even aggravated the upregulation of phosphorylation level of PKCζ by LPS.

Next, we investigated eNOS expression and found that it was decreased after LPS treatment [37] (Fig. 1f, g). Overexpression of SSeCKS (Ad-SSeCKS) was found to counteract the negative effects of LPS on steady laminar flow (s-flow)-induced eNOS expression [38] (Fig. 1f, g).

The results of these data indicate that LPS-induced SSeCKS functions as a negative feedback mechanism to limit endothelial dysfunction in HUVECs.

SSeCKS Inhibits PLS-induced Endothelial PKCζ/ERK5 Dimerization, Reverses PKCζ-mediated Reduction of ERK5 Transcriptional Activation and KLF2-eNOS Expression

First, we examined whether LPS could increase PKCζ/ERK5 dimerization. We found that it significantly heightened PKCζ/ERK5 dimerization (Fig. 2a, b). By contrast, s-low downregulated PKCζ/ERK5 dimerization (Fig. 2c, d). Second, to investigate the involvement of endogenous SSeCKS on LPS-induced PKCζ/ERK5 dimerization, we depleted SSeCKS expression by shRNA in HUVECs. We found that depletion of SSeCKS increased the baseline level of PKCζ/ERK5 dimerization and LPS-induced PKCζ/ERK5 dimerization was significantly amplified in HUVECs (Fig. 2e, f). We also performed a gain-of-function study and found that overexpression of SSeCKS limited the LPS effect on PKCζ/ERK5 dimerization (Fig. 2g, h), suggesting the critical role of SSeCKS in LPS -induced PKCζ/ERK5 dimerization.

Given that PKCζ/ERK5 dimerization by pro-inflammatory cytokine decreased ERK5 transcriptional activity and consequent expressions of KLF2 and eNOS [22], we determined the effects of SSeCKS depletion on ERK5 transcriptional activity. In our study, ERK5 transcriptional activity was decreased by LPS; however, it was further decreased by SSeCKS shRNA (Fig. 2i). Next, we investigated KLF2 and eNOS expression and found that they were decreased after LPS treatment (Figs. 1g, 2j). SSeCKS depletion partly inhibited KLF2 expression under the static condition, especially after LPS treatment (Fig. 2j).

SSeCKS Reduces PKCζ/ERK5 Dimerization

Because SSeCKS inhibits PKC activity by binding to PKCζ [7] and PKCζ binds ERK5 to inhibit ERK5 transcriptional activity [22], we explored whether SSeCKS can reduce PKCζ/ERK5 dimerization via binding PKCζ. After SSeCKS expression in 293T cells, we found decreased association between PKCζ and ERK5 (Fig. 3a). When cells were transfected with SSeCKS and PKCζ, ERK5 was observed to co-precipitate only with PKCζ but almost not with SSeCKS (Fig. 3a). We also coexpressed Myc/DDK-tagged SSeCKS and Myc/DDK-tagged PKCζ with ERK5 and found decreased PKCζ/ERK5 association (Fig. 3a).

To test whether SSeCKS could mediate PKCζ/ERK5 complex formation, we transfected 293T cells with ERK5 and Flag–PKCζ along with increasing amounts of SSeCKS (Fig. 3b). We found that ERK5 was deficient, relative to SSeCKS, in complex formation with PKCζ (Fig. 3b).

Previous findings indicate that PKCζ phosphorylates ERK5 at the phosphorylation site S486 [22]. In our study, ERK5 transcriptional activity was decreased by LPS, but it was further decreased by SSeCKS shRNA (Figs. 2I, 3c). However, ERK5 mutant (S486A) was resistant to the inhibitory effect of SSeCKS shRNA (Fig. 3c). In cells transduced with Ad.ERK5-WT, the transcriptional activity of ERK5 decreased after LPS treatment. By contrast, in cells expressing the Ad.ERK5-S486A mutant, the downregulation of transcriptional activity of ERK5 had been improved after LPS treatment (Fig. 3c).

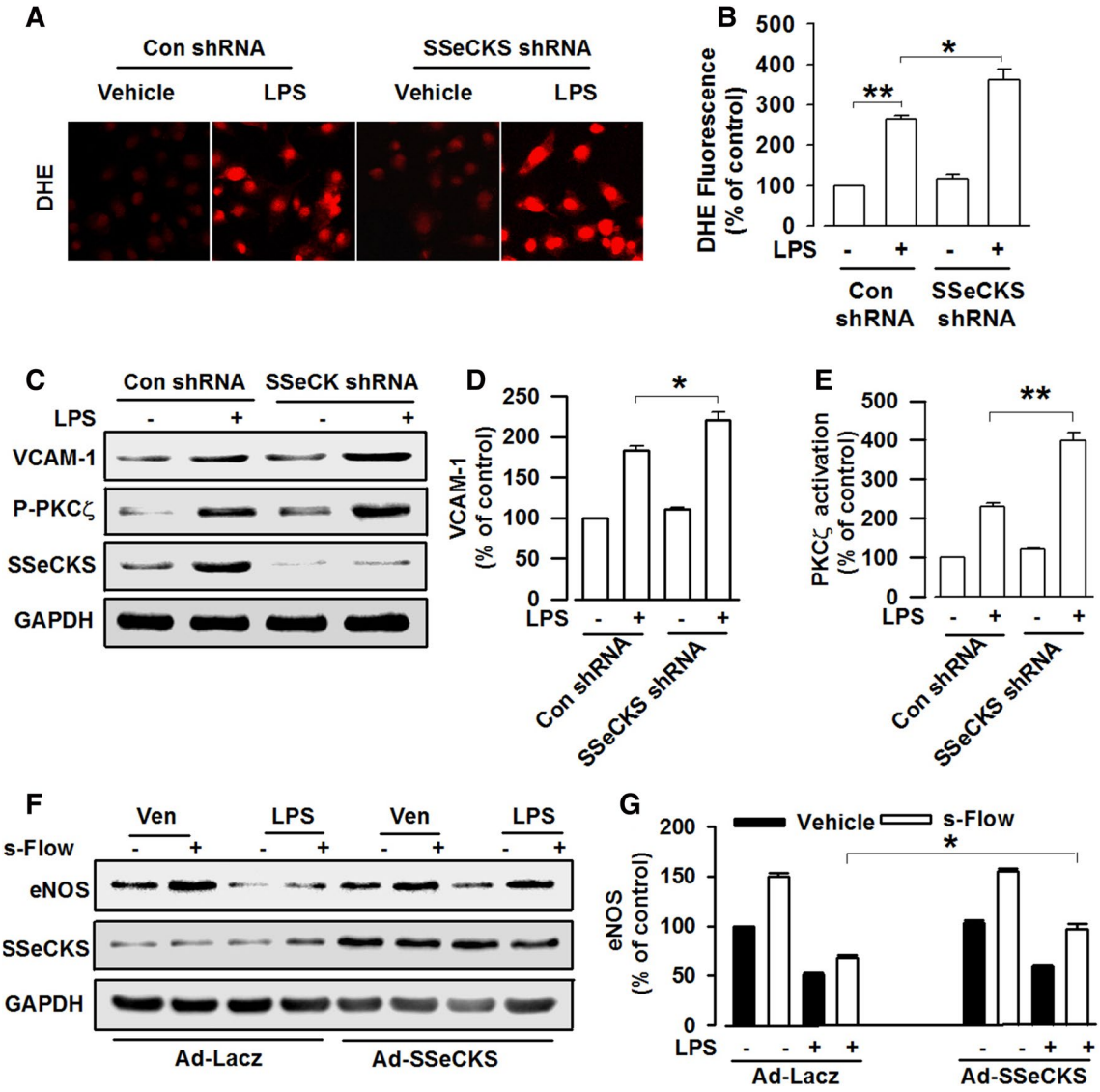
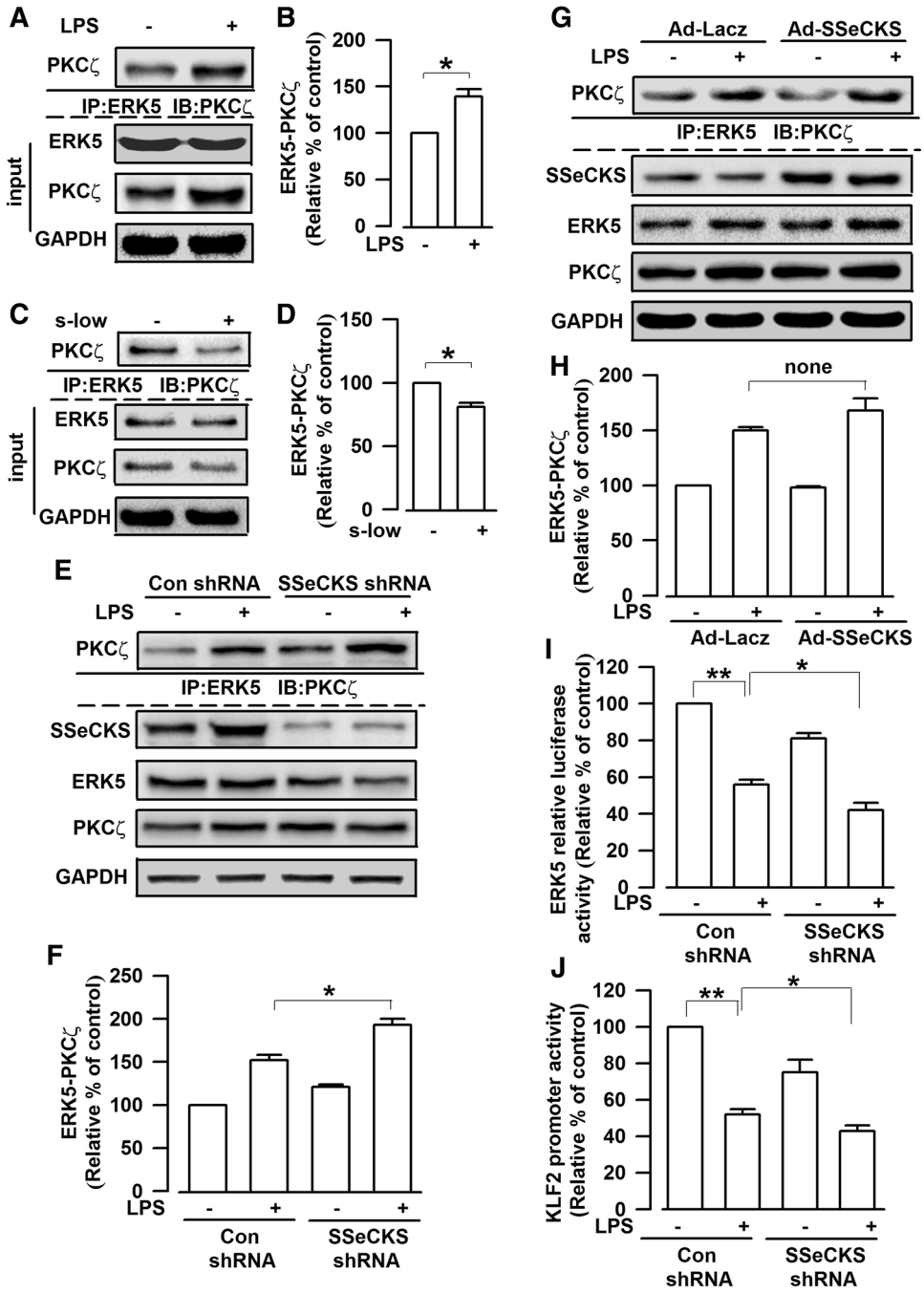


Fig. 1 SSeCKS attenuated superoxides, inhibits adhesion molecule expression, regulates PKCζ activity, and enhances eNOS expression in HUVECs. **a–e** HUVECs were transfected with control or SSeCKS shRNAs for 48 h and then treated with or without LPS for 6 h. DHE superoxide staining **a** was performed to determine ROS production (O_2^- levels). **a, b** O_2^- levels were significantly increased in HUVECs treated with LPS as compared with those treated without LPS. SSeCKS depletion enhanced LPS-mediated upregulation of ROS production. **c, d** VCAM-1 expression was increased in HUVECs treated with LPS as compared with those treated without LPS. SSeCKS depletion enhanced LPS-mediated upregulation of VCAM-1 expression. **c**, e The phosphorylation level of PKCζ was increased in HUVECs treated with LPS as compared with those treated without LPS. SSeCKS depletion enhanced LPS-mediated upregulation of the phosphorylation level of PKCζ. **f, g** HUVECs were transduced with either adenovirus vector containing Lacz (Ad- Lacz) or SSeCKS (Ad-SSeCKS). After 24 h of transduction, cells were treated with or without LPS (30 ng/ml) and then exposed to s-flow for 24 h. eNOS expression was decreased in HUVECs treated with LPS as compared with those untreated with LPS. SSeCKS reversed LPS-mediated downregulation of eNOS expression. $^{*}p < 0.05$, $^{**}p < 0.01$. Results are given as the mean ± SEM of three independent experiments



These results suggest that LPS-mediated downregulation of transcriptional activity of ERK5 depends on PKCζ-mediated ERK5 (S486) phosphorylation, and SSeCKS can reduce PKCζ/ERK5 dimerization via binding PKCζ.

SSeCKS Depletion Aggravates LPS-Mediated Endothelial Dysfunction

To illustrate the role of SSeCKS in endothelial dysfunction after LPS treatment, SSeCKS was silenced using SSeCKS shRNAs via lentiviral transfection. C57BL/6 mouse aortic segments were studied after LPS treatment ex vivo. When the PE-induced contraction reached a plateau in organ chambers, ACh (10^{-9} M–10^{-5} M) or SNP (10^{-9} M–10^{-5} M) was added cumulatively. The capability of the concentration-dependent relaxation induced by ACh, with the maximum response at 10^{-5} M, was weaker in LPS-treated aortic segments than those from control (Fig. 4a). However, the capability of ACh-induced relaxation in the LPS-treated aortic segments of SSeCKS silencing was much weaker than the LPS-treated ones. The capability of the relaxation caused by SNP (10^{-9} M–10^{-5} M) had no difference in the aortic

◂**Fig. 2** SSeCKS inhibits PLS-induced endothelial PKCζ/ERK5 complex formation, reverses PKCζ-mediated reduction of ERK5 transcriptional activation and KLF2-eNOS expression. **a, b** Western blot of anti-ERK5 IP probed with PKCζ antibody, on lysates prepared from HUVECs treated with or without LPS for 6 h. (Lower) Total input probed with indicated antibodies. LPS significantly increased endogenous PKCζ/ ERK5 association. **c, d** Western blot of anti-ERK5 IP probed with PKCζ antibody, on lysates prepared from HUVECs treated with or without s-low for 3 h. (Lower) Total input probed with indicated antibodies. s-low significantly decreased endogenous PKCζ/ ERK5 association. **e, f** HUVECs were transfected with control or SSeCKS shRNAs for 48 h and then treated with or without LPS for 6 h. Western blot of anti-ERK5 IP probed with PKCζ antibody, on lysates prepared from HUVECs. (Lower) Total input probed with indicated antibodies. PKCζ/ ERK5 association was increased in HUVECs treated with LPS as compared with those treated without LPS (n = 20). SSeCKS depletion enhanced LPS-mediated upregulation of PKCζ/ ERK5 association. **g, h** HUVECs were transduced with either adenovirus vector containing Lacz (Ad- Lacz) or SSeCKS (Ad-SSeCKS). After 24 h of transduction, cells were treated with or without LPS (30 ng/ml) and then exposed to s-flow for 3 h. PKCζ/ ERK5 association was increased in HUVECs treated with LPS as compared with those treated without LPS. Western blot of anti-ERK5 IP probed with PKCζ antibody, on lysates prepared from HUVECs. (Lower) Total input probed with indicated antibodies. SSeCKS reversed the upregulation of PKCζ/ERK5 association. **i, g** HUVECs were transfected with control or SSeCKS shRNAs for 48 h and then treated with or without LPS for 6 h. LPS inhibited ERK5 transcriptional activity and KLF2 promoter activity, and SSeCKS depletion enhanced LPS-mediated reduction in ERK5 transcriptional activity and KLF2 promoter activity. Total input probed with indicated antibodies. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{none}p > 0.05$. Results are given as the mean ± SEM of three independent experiments

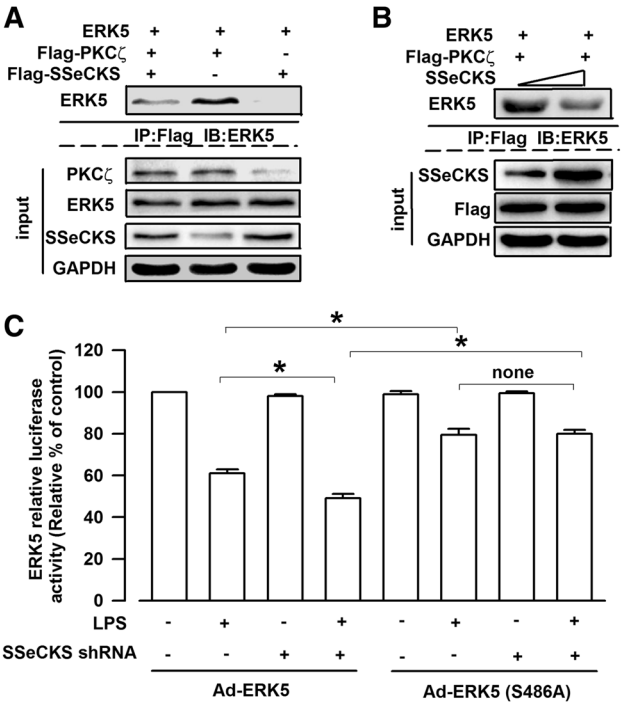


Fig. 3 SSeCKS reduces PKCζ/ERK5 complex formation. **a** Western blot of anti-Myc/DDK IP probed with anti–ERK5 antibody, on lysates prepared from 293T cells transfected with indicated expression or empty vectors. (Lower) Total input probed with indicated antibodies. ERK5 associated with Myc/DDK-tagged PKCζ, in contrast to Myc/DDK-tagged SSeCKS. When Myc/DDK-tagged SSeCKS and Myc/DDK-tagged PKCζ were coexpressed with ERK5, PKCζ/ERK5 association was decreased. **b** Western blot of anti-Myc/DDK IP probed with anti-ERK5 antibody, on lysates prepared from 293T cells cotransfected with equivalent amounts of Myc/DDK-tagged PKCζ and ERK5, and increasing amounts (0.5–1.5 μg) of SSeCKS expression vectors. (Lower) Total input probed with indicated antibodies. When cells were cotransfected with ERK5, Myc/DDK-tagged PKCζ and increasing amounts of SSeCKS, SSeCKS decreased the association between Myc/DDK-tagged PKCζ and ERK5. **c** HUVECs were transduced with either Ad-WT-ERK5 or Ad-ERK5(S496A) mutant with SSeCKS shRNAs or control shRNAs and for 24 h later, treated with or without LPS for 6 h. ERK5 transcriptional activity was assayed by a dual-luciferase reporter assay. $^{*}p < 0.05$, $^{**}p < 0.01$. Results are given as the mean ± SEM of three independent experiments

segments among all groups (Supplementary Fig. 3). In addition, ROS production measured by en face DHE fluorescence in the endothelium of aortic segments was increased by SSeCKS shRNAs (Fig. 4b, c). Ex vivo adenovirus SSeCKS-shRNA transduction blocked the expression of SSeCKS in C57BL/6 mouse aortae (Fig. 4d). The expression of VCAM-1 and the phosphorylation levels of PKCζ were remarkably enhanced by SSeCKS shRNA (Fig. 4d, f). In present study, the maximum constriction of PE of the aortic rings from the LPS group was lower than that of the aortic rings from the control group. However, no significant difference was found between the LPS group and LPS + shRNA-SSeCKS group. (Supplementary Fig. 6)

Discussion

The present study yielded the following novel findings: (i) SSeCKS attenuated superoxides, counteracted inflammation, inhibited PKCζ activity, and enhanced eNOS expression in HUVECs; (ii) SSeCKS–PKCζ binding inhibited LPS-induced endothelial PKCζ/ERK5 dimerization and reversed PKCζ-mediated reduction of ERK5 transcriptional activation and KLF2-eNOS expression; (iii) SSeCKS improved LPS-induced endothelial dysfunction. These results suggest that LPS-induced SSeCKS expression is a negative feedback mechanism to limit PKCζ-mediated reduction of ERK5 transactivation by reducing PKCζ/ERK5 association via binding PKCζ (Supplementary Fig. 4).

S-flow activates ERK5 transcriptional activation [39], which inhibits inflammation [40]. And, the expression of KLF2, a recently identified transcriptional activator of eNOS and an inhibitor of ECs inflammation, can be induced by the s-flow-mediated ERK5 activation [41]. PKCζ participates in inflammatory response through NF-κB activation and LPS-mediated inflammatory response is dependent on

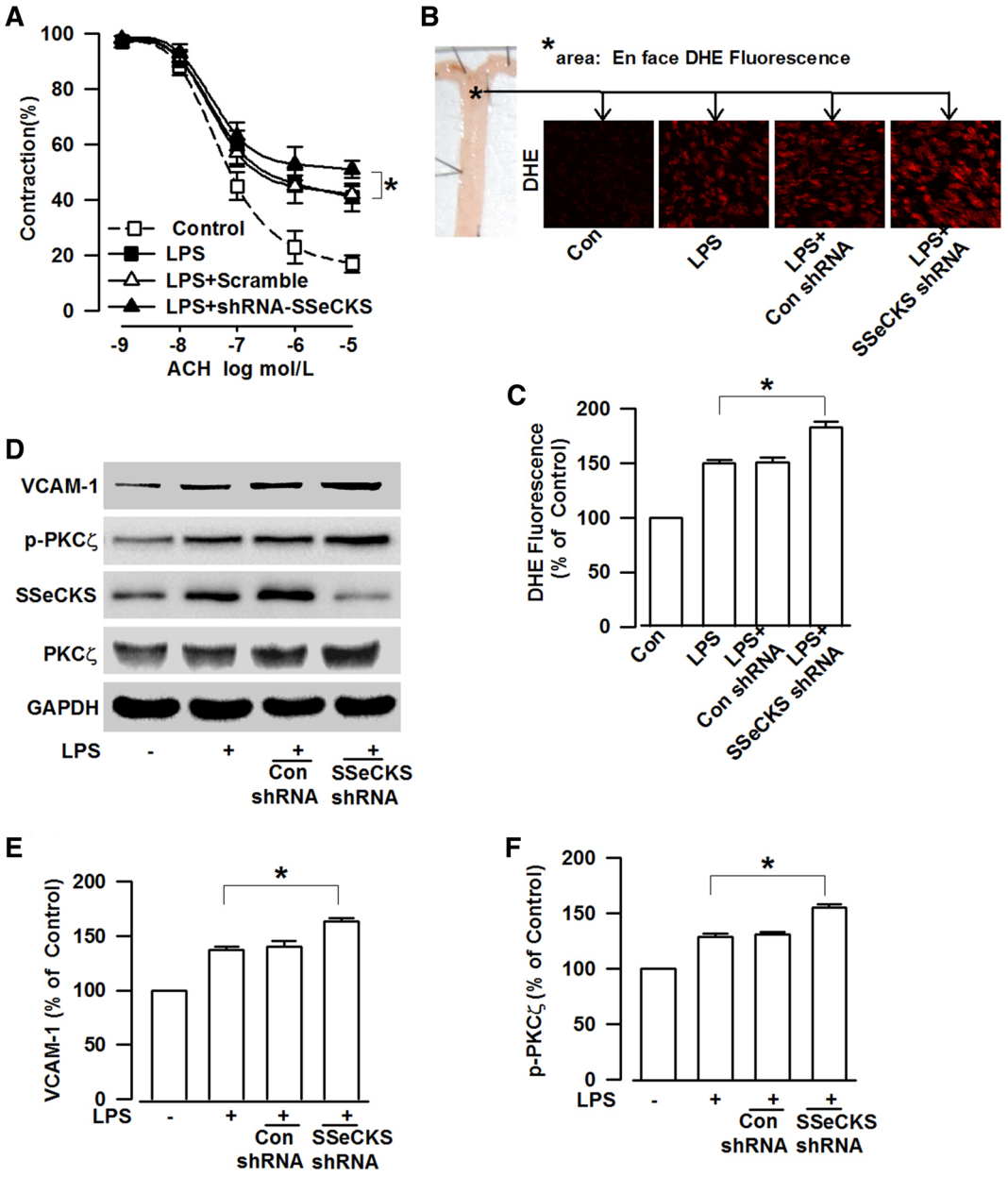


Fig. 4 SSeCKS improves LPS-induced endothelial dysfunction. a-f C57BL/6 mouse aortae were transfected with control or SSeCKS shRNAs for 48 h and then treated with or without LPS for 6 h in ex vivo. a ACh-induced endothelium-dependent relaxations in C57BL/6 mouse aortae treated with LPS as compared with those treated without LPS. SSeCKS depletion enhanced LPS-mediated reduction of vasorelaxation. n = 6–12 rings from 5 to 8 rats. **b, c** DHE superoxide staining was performed to determine ROS production. ROS production was significantly increased in the aortae treated with LPS as compared with those treated without LPS. SSeCKS depletion enhanced LPS-mediated upregulation of ROS production. **d, e** VCAM-1 expression was increased in HUVECs treated with LPS as compared with those treated without LPS. SSeCKS depletion enhanced LPS-mediated upregulation of VCAM-1 expression. **d, f** The phosphorylation level of PKCζ was increased in HUVECs treated with LPS as compared with those treated without LPS. SSeCKS depletion enhanced LPS-mediated upregulation of the phosphorylation level of PKCζ. $^{*}p < 0.05$. Results are given as the mean ± SEM of three independent experiments

PKCζ activation. PKCζ can bind directly to ERK5 and inhibits its ERK5 transcriptional activity via S486 phosphorylation [22]. The PKCζ/ERK5 association inhibits eNOS expression and increases the inflammatory process of ECs [22]. In our study, s-flow also decreased PKCζ/ERK5 association (Fig. 2c, d). PKCζ/ERK5 association was increased in ECs treated with LPS compared with those treated without LPS (Fig. 2g, h). SSeCKS depletion enhanced LPS-mediated upregulation of PKCζ/ERK5 association (Fig. 2e, f). At the same time, the overexpression of SSeCKS did not

considerably enhance LPS-mediated upregulation of PKCζ/ERK5 association (Fig. 2g, h). SSeCKS is a major PKC substrate, which is required to scaffold activated PKC isozymes to sites of activity [2]. However, little is known about the complex interactions among SSeCKS, PKCζ, and ERK5 in vascular endothelium.

LPS results in a pro-inflammatory response, which contributes to ROS production, inflammatory process, and endothelial dysfunction, through activation of PKCζ [42]. LPS also can upregulate the expression of SSeCKS in ECs [5]. However, our results indicate that silencing SSeCKS not only aggravated LPS-induced ROS expression (Figs. 1a, b, 4b, c) but also increased LPS-induced upregulation of VCAM-1 expression (Figs. 1c, d, 4d, f) in vascular endothelium. Furthermore, LPS increased the phosphorylation of PKCζ (Figs. 1c, e, 4d, f), which was also significantly enhanced by silencing SSeCKS (Figs. 1c, e, 4d, f). Taken together, these data suggest that SSeCKS association with PKCζ acts as a negative feedback to inhibit PKCζ activity.

Our findings show that SSeCKS might function as a scaffolding protein to inhibit PKCζ signaling as suggested by the detection of PKCζ in complex with ERK5. PKCζ binds directly to ERK5 [22]. We found that ERK5 S486A mutant was resistant to the inhibitory effect of LPS (Fig. 3c), demonstrating that PKCζ regulates ERK5 transcriptional activity via S486 phosphorylation. This finding is consistent with the observations of Patrizia et al. [22], suggesting that the overexpression of SSeCKS could influence the ability of PKCζ to bind and phosphorylate ERK5, which may be important to decrease eNOS protein stability and contribute to endothelial dysfunction [22]. Last, we found that SSeCKS improves LPS-induced endothelial dysfunction (Fig. 4a). So, to uncover a novel regulatory mechanism of SSeCKS for preventing LPS-induced vascular dysfunction, our study has two important implications: ① LPS-induced SSeCKS functions as a negative feedback mechanism to directly scaffold PKCζ in ECs and desensitize PKCζ activity; ② PKCζ-binding domains in SSeCKS are required to inhibit LPS-induced PKCζ/ERK5 complex formation in ECs.

In summary, the results suggest that the expression of SSeCKS induced by LPS, as a negative feedback mechanism, has the potential to improve endothelium-dependent relaxation in vascular disease conditions by inhibiting PKCζ-mediated reduction of ERK5 transactivation. However, the role of SSeCKS in vascular health and its functions or molecular mechanisms need further investigation.

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Compliance with Ethical Standards

Conflict of interest No conflict of interest has been declared.

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