Mol Cell Biochem (2007) 305:1–8
DOI 10.1007/s11010-007-9521-7

Lipopolysaccharide induces expression of SSeCKS in rat lung microvascular endothelial cell

Chun Cheng · Haiou Liu · Haiyan Ge · Ji Qian · Jing Qin · Linlin Sun · Menling Chen · Meijuan Yan · Aiguo Shen

Received: 16 February 2007 / Accepted: 16 May 2007 / Published online: 6 June 2007

Abstract *Src*-suppressed C kinase substrate (SSeCKS) plays a role in membrane-cytoskeletal remodeling to regulate mitogenesis, cell differentiation, and motility. Previous study showed that lipopolysaccharide (LPS) induced a selective and strong expression of SSeCKS in the vascular endothelial cells of lung. Here we show that LPS stimulation elevated expression of SSeCKS mRNA and protein in Rat pulmonary microvascular endothelial cell (RPMVEC). LPS potentiated SSeCKS phosphorylation in a time- and dose-dependent manner, and partly induced translocation of SSeCKS from the cytosol to the membrane after LPS challenge. The PKC inhibitor, Calphostin C, significantly decreased LPS-induced phosphorylation of SSeCKS, inhibited SSeCKS translocation and actin cytoskeleton reorganization after LPS challenge, suggesting that PKC may play a role in LPS-induced SSeCKS translocation and actin rearrangement. We conclude that SSeCKS is located downstream of PKC and that SSeCKS and PKC are both necessary for LPS-induced stress fiber formation.

Keywords Rat pulmonary microvascular endothelial cell · Lipopolysaccharide · SSeCKS · Actin

Chun Cheng and Haiou Liu are contributed equally to this work.

C. Cheng · H. Liu
Institute of Nautical Medicine, Nantong University (former Nantong Medical College), Nantong 226001, P.R. China

H. Ge · J. Qian · J. Qin · L. Sun · M. Chen
Medical College, Nantong University (former Nantong Medical College), Nantong 226001, P.R. China

M. Yan · A. Shen (✉)
Jiangsu Province Key Laboratory of Neuroregeneration, Nantong University (former Nantong Medical College), Nantong 226001, P.R. China
e-mail: shen_aiguo@yahoo.com

Introduction

The endothelial cell (EC) lining of blood vessels provide integrity and a selective barrier to fluid and solute flux across the vascular wall. Breakdown of this barrier by bacterial endotoxin (lipopolysaccharide, LPS) during sepsis results in increased EC monolayer permeability in the lungs and development of adult respiratory distress syndrome (ARDS). It is well-known that barrier integrity is critically dependent on the cytoskeleton that regulates actin stress fiber formation, cell shape, and cellular adherence [1, 2]. Morphological studies of LPS-exposed pulmonary EC monolayers have demonstrated actin reorganization, intercellular gap formation, and increased permeability [3, 4]. However, the signal transduction mechanisms for LPS-induced EC microfilament reorganization are not clear.

Activation of protein kinase C (PKC) is a common pathway through which LPS cause endothelial cell cytoskeleton reorganization [5, 6]. In many cell types, activated PKC directly promotes cytoskeletal reorganization through phosphorylation of proteins at plasma membranes sites, such as the focal adhesion proteins vinculin and tensin [7–9]. Other reports suggest that activated PKC may control cytoskeletal architecture by dominating over other signaling pathways, such as RhoA-mediated stress fiber formation [10, 11]. Yet, little information exists on downstream targets of PKC in rat pulmonary microvascular endothelial cell (RPMVEC).

Src-suppressed C kinase substrate (SSeCKS) has a motif for binding PKC/PKA and is phosphorylated by PKC [12]. Several in vitro studies using cell lines have demonstrated

that SSeCKS actually scaffolds PKC and PKA and is involved in the regulation of actin-based cytoskeletal dynamics and the cell cycle [13–15]. Previous in vivo studies demonstrated that SSeCKS is expressed in some restricted types of cells, such as spermatids at the late stage of spermatogenesis and renal mesangial cells, suggesting a role for SSeCKS in membrane—cytoskeletal remodeling and cell motility [16–18]. The SSeCKS localizes to membrane and cortical cytoskeletal sites in fibroblasts, and overexpression of SSeCKS causes a profound change in cellular morphology [12]. These combined data suggest a potential role of SSeCKS in highly coordinated series of cytoskeletal remodeling in various cell systems.

It was recently reported that bacterial lipopolysaccharide potently induces SSeCKS expression in the endothelium of murine blood vessels. Cells expressing SSeCKS after a LPS challenge included endothelial cells of the liver, lung, heart, and brain [19]. Furthermore, the involvement of SSeCKS in the uptake of particles is suggested by the elevated uptake by hepatic endothelial cells and lymphoid reticular cells when SSeCKS expression was induced by LPS administration [20]. To date, there is little information about changes of SSeCKS in endothelial cells stimulated by LPS in vitro. Therefore, in the present study we chose RPMVECs to determine, whether the expression level and location of SSeCKS could be affected in these cells by treatment with LPS.

Materials and methods

Materials

LPS from *Escherichia coli* (055:B5), Calphostin C, polyclonal anti-SSeCKS antibody, fluorescein isothiocyanate (FITC)-conjugated Bandeiraea simplicifolia I isolectin B4 (BSI), and fluorescein isothiocyanate (FITC)-conjugated phalloidin were all purchased from Sigma (St. Louis, MO, USA). Rhodamin-conjugated anti-sheep antibody was purchased from Jackson laboratory (Bar Harbor, ME). Trizol Isolation Reagent and High glucose Dulbecco's modified Eagle's medium (DMEM) were all purchased from invitrogen (Carlsbad, CA). Fetal calf serum, penicillin, and streptomycin were purchased from HyClone Laboratories (Salt Lake city, UT). Plastic tissue culture flasks were purchased from Costar (Costar, Cambridge, MA).

Cell culture

Isolation and culture of (Rattus pulmonary microvessel endothelial cells) RPMVEC has been described previously [21]. The culture medium contained DMEM (GIBCO BRL; Grand Island, NY) supplemented with 20% fetal bovine serum (HyClone Laboratories; Logan, UT), 15 μg/ml endothelial cell growth supplement (Upstate Biotechnology; Lake Placid, NY), and 1% nonessential amino acids (GIBCO BRL). Pulmonary microvascular endothelial monolayers were maintained in 5% CO2 plus humidified air at 37°C and reached confluence within two to three population doublings, which took 4–5 days. The preparations were identified as endothelial monolayers by (1) the characteristic ''cobblestone'' appearance using contrast microscopy, (2) the presence of platelet endothelial cell adhesion molecule (PECAM-1) antigen (indirect immunofluorescence), (3) the binding of Lectin BSI.

Binding of lectin BSI

The fluorescently labeled lectin BSI binds primarily to microvascular endothelial cells and does not bind to large vessel endothelial cells or most other control non-endothelial cells [22, 23]. Monolayers of RPMVECs were grown to confluence on gelatin-coated glass coverslips. The coverslips were then washed two times with PBS, fixed in acetone at 4°C, and allowed to air dry. The cells were then incubated with 25 μg/ml of FITC-labeled BSI for 30 min at room temperature. Subsequently, cells were washed three times with PBS and viewed with a fluorescence microscope.

Real-time PCR

Total RNA (1 μg) extracted from RPMVECs was used as a template for cDNA synthesis. cDNA was prepared by use of a ominiscript RT Kit (Qiagen, Chatsworth,CA). Real-time PCR (performed on three independent pooled samples) was performed on a Rotor-Gene 2000 (Corbett Research, Sydney, Australia). TaqMan GAPDH Control Reagents were used as internal control for normalization. Quantification primers and probes were designed using Primer Express. For SSeCKS (GenBank accession no. AY695056): sense, 5′-AAGTGCTGGCTTCGGAGAAAG-3′ (3106–3126); antisense, 5′-TGACTTCAGGAACTTCAAGGCTC-3′ (3215–3237); probe, 5′-AGCCTGTCCAGTCTCAGAGCCCTGTG-3′ (3206–3181). All the probes were labeled with 6-carboxytetramethyl-rhodamine at the 3′-end and FAM at the 5′-end for target and GAPDH, respectively. Fluorescent signals were generated during each PCR cycle via the 5′–3′-endonuclease activity of TaqMan Probe to provide specific transcript expression levels. For the reaction, PCR cycle parameters were 40 cycles of 94°C for 15 s, 60°C for 45 s. Threshold cycle (Ct), which correlates inversely with the target mRNA levels, was set as the cycle at which each fluorescent signal

and was first detected above background. Initial experiments demonstrated identical realtime amplification efficiencies of target and reference gene which is a prerequisite for the relative quantification, 2-ΔΔC t method, used here. The expression level of each target gene was calculated by standardizing the target gene copy number with the GAPDH copy number in a sample. Purity and specificity of all products were confirmed by omitting the template and by appropriate size and single-melting temperature. Analysis of results is based on three independent experiments. Specific mRNA transcript levels were expressed as fold difference.

Immunoprecipitation and Western Blotting

Cells were washed with ice-cold phosphate-buffered saline and lysed (0.5% Triton, 50 mM NaCl, 10 mM NaF, 30 mM tetrasodium pyrophosphate, 10% glycerol, 1 mM EDTA, 20 mM Tris, 1 mM Pefabloc, 1% Trasylol, 1 mM sodium orthovanadate, pH 7.4). Extracts were clarified by centrifugation, and protein concentration was determined by the BCA protein assay system (Pierce). Equal amounts of lysates were incubated with 1 μg of antibody as indicated. The immunoprecipitates were collected on protein A-Sepharose beads, washed three times in lysis buffer, and then boiled in SDS sample buffer containing dithiothreitol. Immunoprecipitates or equal amounts of total cell lysates were analyzed by SDS-PAGE. For Western blotting, samples were electrotransferred to polyvinylidene difluoride membranes (Immobilon P), which were blocked in 5% bovine serum albumin in phosphate-buffered saline solution containing 0.1% Tween 20. Primary antibodies were used with the concentrations and buffers recommended by the suppliers and incubated overnight in the cold. After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (Amersham Biosciences), Finally, the blots were visualized with enhanced chemiluminescence.

Cell fractionation analysis

Cellular fractionations to assess the distribution of endogenous SSeCKS was performed as previously described [24]. A quantity of 80 mg of protein from the cytosolic or membrane fractions were loaded on 6% SDS-polyacrylamide gels, and levels of protein in each fraction were determined by western blot analysis.

Immunofluorescence

Primary RPMVECs were seeded onto sterile 22 mm^2 coverslips at a density of approximately 50% and allowed to grow for 18–24 h. In some cases, cells were treated with 1μg/ml LPS, in the presence or absence of 0.05 μM/l Calphostin C before fixing. The cells were fixed at 4°C for 20 min with precooled 3.7% formaldehyde and extracted with 0.1% Triton X-100 for 10 min and blocked by 1%BSA for 2 h. After washing in PBS, the cells were incubated for 1 h with sheep polyclonal anti-SSeCKS antibody at a 1:250 dilution. Cells were washed with PBS 5 times and incubated with rhodamine-conjugated anti-sheep IgG or fluorescein-labeled phalloidin for 30 min. Finally, the cells were washed with PBS and mounted on coverglass with glycerin and PBS (1:1). The cells were examined under a Zeiss Confocal Laser Scanning Microscope and fluorescence microscope.

Statistical analysis

All experiments were repeated at least three times. All numerical data were expressed at mean ± SD. Data were analyzed using the two-tailed *t* test. A probability value of 0.05 or less was considered significant. In some cases, S.D. values were too low to appear in the graph as error bars.

Results

RPMVECs characteristics

RPMVECs grew in monolayers with morphology consistent with endothelial cells by phase-contrast microscopy were examined (Fig. 1A, B). The cells grew initially as capillary-like structures and assumed typical cobblestone morphology of endothelial cells at confluence (Fig. 1A). These cells were characterized as endothelial cells by PECAM-1 expression (Fig. 1C). Meanwhile, the cells demonstrated intense lectin binding criteria for microvascular endothelial cells. Figure 1D showed that the RPMVECs, grown on coverslips, bound FITC-labeled BSI and displayed positive staining by lectin BSI.

Up-regulation of SSeCKS mRNA expression by LPS

To assess the influence of LPS on RPMVECs SSeCKS mRNA expression, the RPMVEC were stimulated with LPS (1–1000 ng/ml) for 1 h, in a dose-dependent increase in expression of SSeCKS mRNA as shown in Fig. 2A. Next we examined time course of SSeCKS mRNA upregulation by using 1 μg/ml of LPS. Cells were stimulated with LPS for various courses, and SSeCKS mRNA expression was examined. LPS increased SSeCKS mRNA expression occurred at 0.5, 1, and 3 h of stimulation, respectively, (Fig. 2B).

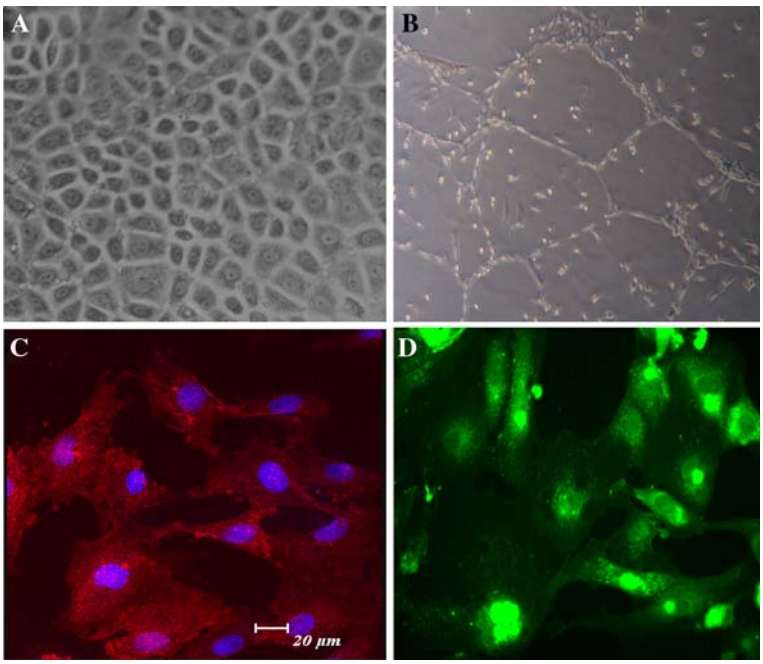

Fig. 1 Rat pulmonary microvascular endothelial cells (RPMVECs) morphology, PECAM-1 expression and binding of Lectin BSI. (**A**, **B**) Normal RPMVECs morphometrics under phase-contrast microscopy (magnification ×200), (**C**) RPMVECs were labeled with anti- PECAM-1, (**D**) RPMVECs bound to FITC-labeled BSI under fluorescence microscopy'(magnification ×200)

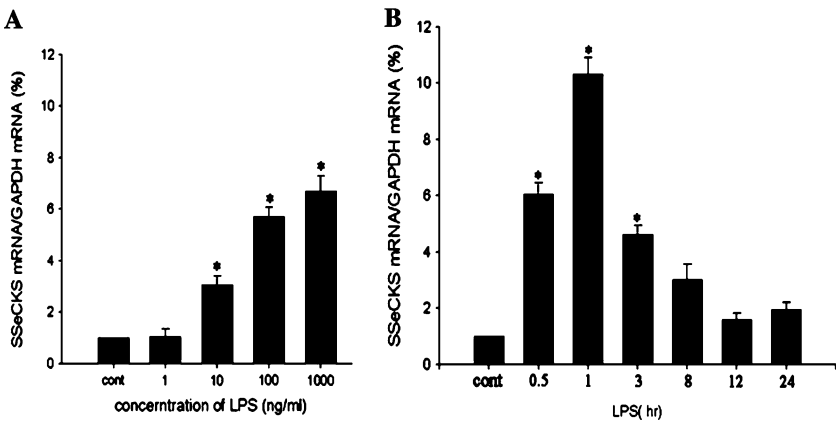

Fig. 2 Lipopolysaccharide (LPS)-induced SSeCKS mRNA expression in RPMVEC. RPMVEC were incubated with increasing concentrations of LPS for 1 h (**A**), LPS (1 μg/ml) or media alone for increasing exposure times (**B**)

The SSeCKS mRNA increase is accompanied by a similar increase in SSeCKS protein levels

We sought to determine whether the protein increase was associated with a corresponding elevation of SSeCKS mRNA. Figure 3A shows that the levels of SSeCKS protein prepared from cells that had been treated with LPS (1–1000 ng/ml) for 3 h were elevated at dose-dependent manner; the elevated of SSeCKS protein level after LPS challenge was similar in extent to that of the SSeCKS mRNA. The increase in SSeCKS protein peaked at the 3 h of LPS treatment, a few hours late the peak of the SSeCKS mRNA (Fig. 3B). When pretreatment with the protein synthesis inhibitor cycloheximide, there was little increase in SSeCKS expression after LPS challenge (Fig. 3C). The inhibitory effect of cycloheximide is consistent with the previous results that LPS-induced SSeCKS expression.

LPS induced SSeCKS phosphorylation

SSeCKS binds PKC in a phosphatidylserine-dependent manner and also is a major PKC substrate in vitro and in vivo [12, 13]. As previously shown, activation of PKC by the short-term addition of nanomolar concentrations of phorbol esters is known to result in the rapid phosphorylation of SSeCKS. Since LPS potentiates PKC-dependent signals in the endothelial cell [25], we sought to evaluate the phosphorylation of SSeCKS after LPS treatment. Treatment of RPMVEC with increasing concentrations of LPS led to dosage-dependent increases in SSeCKS phosphorylation (Fig. 4A), reaching maximal levels at 1 μg/ml. LPS induced SSeCKS phosphorylation occurred rapidly (5 min) and reached at peak level at 30 min (Fig. 4B). In order to assess, whether these phosphorylation events stimulated by LPS were PKC-dependent, we pretreated

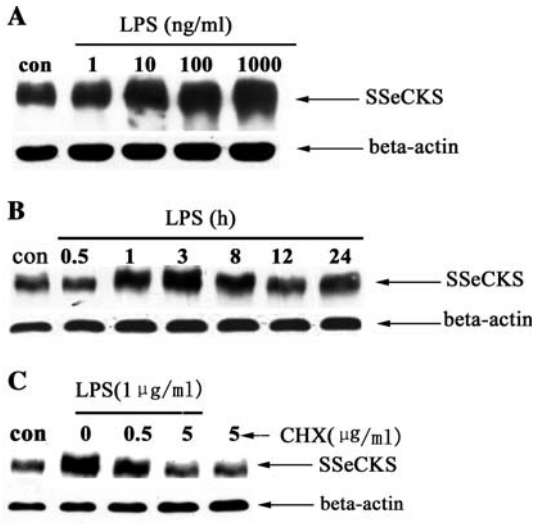

Fig. 3 Lipopolysaccharide (LPS)-induced SSeCKS protein expression in RPMVEC. RPMVEC were incubated with increasing concentrations of LPS for 3 h (**A**), LPS (1 μg/ml) or media alone for increasing exposure times (**B**). RPMVEC were preincubated for 30 min with 0.5 and 5 μg/ml of cycloheximide (CHX), respectively, without medium change. Next, the cells were stimulated with LPS (1 μg/ml) for 3 h and harvested. As negative controls, cells were only treated with the 5 μg/ml of CHX (**C**)

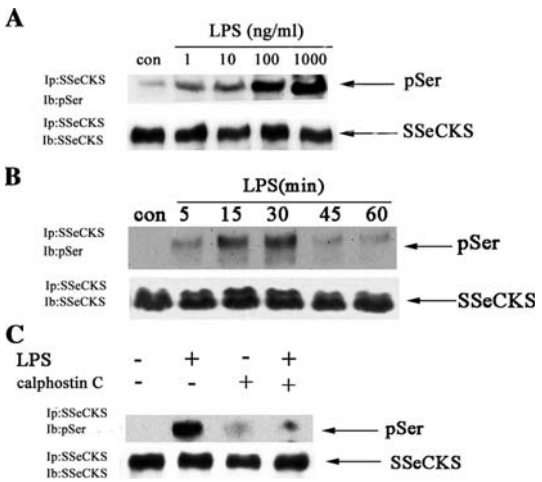

Fig. 4 LPS induced SSeCKS phosphorylation. (**A**) RPMVEC were grown to confluence on 60-mm dishes and treated with increasing concentrations of LPS for 30 min. (**B**) RPMVEC were treated with 1μg/ml LPS for indicated time. (**C**) RPMVEC were pretreatment with 0.05 μM/l Calphostin C for 30 min as described, followed by LPS (1 μg/ml) treatment for 30 min. SSeCKS was immunoprecipitated (Ip) and the filter subjected to immunoblotting (Ib) with antibodies against phosphoserine (pSer) (top panel), SSeCKS (bottom panel)

RPMVECs for 30 min with 0.05μM/l Calphostin C. We found that depletion of PKC activities before LPS treatment resulted in attenuation of SSeCKS phosphorylation (Fig. 4C). These data indicate that activation of PKC is required, at least in part, in phosphorylation of SSeCKS induced by LPS.

LPS induced SSeCKS translocation

Immunofluorescence analysis indicated that SSeCKS localized to the cytoplasm, but was enriched at the cell edge, in structures resembling podosomes (Fig. 5 A). The association of SSeCKS with cortical actin-like structures (Fig. 5A) and cellular components such as podosomes (Fig. 5A–C) further suggested a role for SSeCKS in the control of actin-based cytoskeletal architecture. By 1 h of LPS treatment, after which actin filaments appeared as irregular spikes with more orthogonal filaments in contrast to straight fibers in control RPMVECs and SSeCKS translocated from the cytosol to the membrane cites (Fig. 5D–F). In order to assess the role of PKC in defining SSeCKS and cytoskeleton in RPMVECs in response to LPS, we attempted to ablate activities of PKC by Pan PKC inhibitor Calphostin C. Our results showed that pretreatment with Calphostin C before LPS challenge resulted in thin stress fibers emanating toward cell edges and SSeCKS filamentous staining (Fig. 5G–I). These data clearly show a link between PKC activation, SSeCKS translocation and control of actin cytoskeleton structure in RPMVEC. We employed another method to evaluate the distribution of SSeCKS between cytosol and membrane. Under basal conditions, SSeCKS exist for the most part in the cytosolic fraction (Fig. 6). After LPS treatment for 5–60 min, we saw incomplete translocation of SSeCKS from the cytosol to the membrane (Fig. 6)

Discussion

One of the essential functions of the endothelial cell (EC) lining is to maintain the essentially impermeable nature of the blood vessel, controlling the passage of solutes and inflammatory cells from the circulation to the tissues. EC exposure to LPS initiates a coordinate series of biological responses (shape change, cell migration, or intercellular gap formation), numerous studies have demonstrated PKC-dependent alteration of these EC functions [5, 6]. However, few reports have identified downstream targets of PKC in EC that may mediate these changes in function. Here, we report that SSeCKS, a novel PKC substrate of EC that likely plays a role in EC cytoskeleton reorganization.

Evidence is showing that SSeCKS expression is regulated and involved in important functions [12–18]. In this report, we provide new evidence showing that LPS up-regulates the expression of SSeCKS in endothelial cells. Furthermore, the time- and concentration-dependent in-

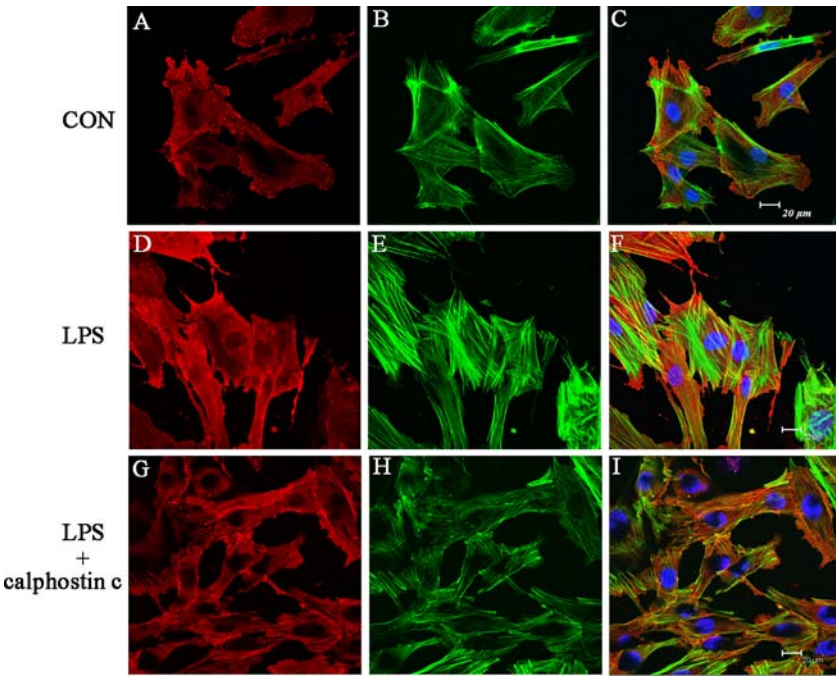

Fig. 5 LPS affected SSeCKS distribution and actin cytoskeleton. RPMVECs were treated with either LPS (1 μg/ml, 1 h) or PKC inhibitor Calphostin C (0.05 μM, 30 min) and then with LPS (1 μg/ml, 1 h). After the treatment the cells were double-stained to visualize SSeCKS simultaneously with actin. Anti-SSeCKS antibodies were used for immunofluorescent detection of SSeCKS. Actin microfilaments were stained with FITC-labeled phalloidin. Double-stained images are shown in parallel. The distribution of SSeCKS and f-actin in control RPMVECs (**A–C**); LPS affected both SSeCKS distribution and f-actin structures (**D–F**); Calphostin C pretreatment partly prevented the effects of LPS on SSeCKS distribution and cytoskeletal structures of RPMVEC (**G–I**)

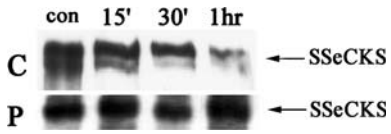

Fig. 6 SSeCKS redistributed between cytosol and membrane after LPS stimulation. Western blot analysis showing SSeCKS redistributed from cytosolic (C) to membrane pellet (P) compartments at various time points after stimulation with LPS(1 μg/ml)

crease in mRNA and protein levels is accompanied by an increase in the cell edge localization of SSeCKS. We also demonstrated that SSeCKS is phosphorylated by LPS in RPMVEC in a time- and dose-dependent manner and that this phosphorylation is inhibited, in part, by a PKC inhibitor, Calphostin C. This study, for the first time, implicates LPS induced SSeCKS translocation partly through PKC-dependent manner.

SSeCKS mRNA expression is regulated by LPS in a time- and concentration-dependent manner, indicative of a direct effect of LPS signaling. The involvement of delayed regulatory systems, such as the synthesis of intermediate regulatory molecules or the effect of secondary cytokines secreted by RPMVECs under the influence of LPS, is excluded by the short-time needed to reach a maximum effect.

The potent induction of SSeCKS by LPS is likely to be due to an accelerated rate of the protein's synthesis, as indicated by the large elevation in SSeCKS mRNA induced by LPS, together with the sensitivity of the SSeCKS increase to protein synthesis inhibitors. The LPS-induced SSeCKS expression in EC has been examined in past studies, but the results obtained were not consistent. Hiroshi Kitamura and colleagues failed to detect any increase of SSeCKS mRNA and protein in cultured murine endothelial cell line LEII, even after LPS stimulation [19]. The controversial findings on the SSeCKS induction by LPS may be attributable to unusual characteristics of this immortal and proliferative cell line. It is worth noting that there is a functional difference between the primary endothelial cell and endothelial cell lines with regard to their response to cytokines [26].

The LPS has been shown to increase the PKC activity in a variety of cells, including EC [27, 28]. Most importantly with regard to our work, a previous study has shown that upon PMA stimulation of fibroblasts and mesangial cells, SSeCKS phosphorylation is mediated in a PKC-dependent fashion [17]. Our study is further evidence of the involvement and possible interaction between SSeCKS and PKC, specifically upon stimulation by LPS.

The results from the present study indicated that after LPS treatment, SSeCKS is enriched at the cell edge. The distribution of SSeCKS in RPMVECs following LPS stimulation was consistent with Toshihiko and colleagues' studies that SSeCKS was localized predominantly along the cytoplasmic membrane of both sinusoidal endothelial cells of the liver and medullary reticular cells of the lymph node after LPS stimulation [20]. Pretreatment with PKC inhibitor, LPS did not seem to change on the cellular distribution of SSeCKS in that SSeCKS was found associated with microfilaments. As was previously demonstrated for SSeCKS associated with F-actin in vivo and in vitro [29, 30]. Altogether, the above results and considerations demonstrate that a marked increase in SSeCKS in RPMVEC after LPS treatment, and is partly required for reorganization of the actin cytoskeleton induced by LPS.

Acknowledgments This work was supported by National Natural Scientific Foundation of China Grant 30300099, and Natural Science Foundation of JiangSu province Grant BK2003035, and Natural science Foundation of JiangSu colleges and universities Grant 03KJB180109 and ''liu-da-ren-cai-gao-feng'' finance assistance of JiangSu province Grant No. 2.

References

1. McVerry BJ, Garcia JG (2004) Endothelial cell barrier regulation by sphingosine 1-phosphate. J Cell Biochem 92:1075–1085
2. Waschke J, Baumgartner W, Adamson RH et al (2004) Requirement of Rac activity for maintenance of capillary endothelial barrier properties. Am J Physiol Heart Circ Physiol 286:H394–H401
3. Chakravortty D, Nanda Kumar KS (2000) Bacterial lipopolysaccharide induces cytoskeletal rearrangement in small intestinal lamina propria fibroblasts: actin assembly is essential for lipopolysaccharide signaling. Biochim Biophys Acta 1500:125–136
4. Goldblum SE, Ding X, Brann TW et al (1993) Bacterial lipopolysaccharide induces actin reorganization, intercellular gap formation, and endothelial barrier dysfunction in pulmonary vascular endothelial cells: concurrent F-actin depolymerization and new actin synthesis. J Cell Physiol 157:13–23
5. Serikov VB, Choi H, Schmiel K et al (2004) Endotoxin induces leukocyte transmigration and changes in permeability of the airway epithelium via protein-kinase C and extracellular regulated kinase activation. J Endotoxin Res 10:55–65
6. Hai CM, Hahne P, Harrington EO et al (2002) Conventional protein kinase C mediates phorbol-dibutyrate-induced cytoskeletal remodeling in a7r5 smooth muscle cells. Exp Cell Res 280:64–74
7. Massoumi R, Sjolander A (2001) Leukotriene D(4) affects localisation of vinculin in intestinal epithelial cells via distinct tyrosine kinase and protein kinase C controlled events. J Cell Sci 114:1925–1934
8. Tatin F, Varon C, Genot E et al (2006) A signalling cascade involving PKC, Src and Cdc42 regulates podosome assembly in cultured endothelial cells in response to phorbol ester. J Cell Sci 119:769–781
9. Harrington EO, Brunelle JL, Shannon CJ et al (2003) Role of protein kinase C isoforms in rat epididymal microvascular endothelial barrier function. Am J Respir Cell Mol Biol 28: 626–636
10. Brandt D, Gimona M, Hillmann M et al (2002) Protein kinase C induces actin reorganization via a Src- and Rho-dependent pathway. J Biol Chem 277:20903–20910
11. Tang S, Morgan KG, Parker C (1997) Requirement for protein kinase C theta for cell cycle progression and formation of actin stress fibers and filopodia in vascular endothelial cells. J Biol Chem 272:28704–28711
12. Lin X, Tombler E, Nelson PJ (1996) A novel src- and ras-suppressed protein kinase C substrate associated with cytoskeletal architecture. J Biol Chem 271:28430–28438
13. Gelman IH, Lee K, Tombler E (1998) Control of cytoskeletal architecture by the src-suppressed C kinase substrate, SSeCKS. Cell Motil Cytoskeleton 41:1–17
14. Lin X, Nelson P, Gelman IH (2000) SSeCKS, a major protein kinase C substrate with tumor suppressor activity, regulates G(1) → S progression by controlling the expression and cellular compartmentalization of cyclin D. Mol Cell Biol 20:7259–7272
15. Erlichman J, Gutierrez JR, Zucker S et al (1999) Developmental expression of the protein kinase C substrate/binding protein (clone 72/SSeCKS) in rat testis identification as a scaffolding protein containing an A-kinase-anchoring domain which is expressed during late-stage spermatogenesis. Eur J Biochem 263:797–805
16. Gelman IH, Tombler E, Vargas JJ et al (2000) A role for SSeCKS, a major protein kinase C substrate with tumour suppressor activity, in cytoskeletal architecture, formation of migratory processes, and cell migration during embryogenesis. Histochem J 32:13–26
17. Nelson PJ, Moissoglu K, Vargas JJ et al (1999) Involvement of the protein kinase C substrate, SSeCKS, in the actin-based stellate morphology of mesangial cells. J Cell Sci 112:361–370
18. Gelman IH, Xi Q, Kumar CC (1999) Reexpression of the major PKC substrate, SSeCKS, correlates with the tumor-suppressive effects of SCH51344 on rat-6/src and rat-6/ras fibroblasts but not on rat-6/raf fibroblasts. Ann N Y Acad Sci 886:221–224
19. Kitamura H, Okita K, Fujikura D, Mori K, Iwanaga T, Saito M (2002) Induction of Src-suppressed C kinase substrate (SSeCKS) in vascular endothelial cells by bacterial lipopolysaccharide. J Histochem Cytochem 50:245–255
20. Rung-ruangkijkrai T, Fujikura D, Kitamura H et al (2004) The expression of src-suppressed C kinase substrate (SSeCKS) and uptake of exogenous particles in endothelial and reticular cells. Arch Histol Cytol 67:135–147
21. Chen S, Fei X, Li SH (1995) A new simple method for isolation of microvascular endothelial cells avoiding both chemical and mechanical injuries. Microvasc Res 50:119–128
22. Hansen-Smith FM, Watson L, Lu DY et al (1988) Griffonia simplicifolia I: fluorescent tracer for microcirculatory vessels in nonperfused thin muscles and sectioned muscle. Microvasc Res 36:199–215
23. Laitinen L (1987) Griffonia simplicifolia lectins bind specifically to endothelial cells and some epithelial cells in mouse tissues. Histochem J 19:225–234
24. Disatnik MH, Boutet SC, Lee CH, Mochly-Rosen D, Rando TA (2002) Sequential activation of individual PKC isozymes in integrin-mediated muscle cell spreading: a role for MARCKS in an integrin signaling pathway. J Cell Sci 115:2151–2163
25. Yang Z, Bochsler PN, Carroll RC et al (1994) Signal transduction pathways of bacterial lipopolysaccharide-stimulated bovine vascular endothelial cells. Inflammation 18:221–233
26. Lidington EA, Moyes DL, McCormack AM et al (1999) A comparison of primary endothelial cells and endothelial cell lines for studies of immune interactions. Transpl Immunol 7:239–246

27. Karimi G, Fatehi Z, Gholamnejad Z (2006) The role of nitric oxide and protein kinase C in lipopolysaccharidemediated vascular hyporeactivity. J Pharm Pharm Sci 9:119–123
28. Chen YJ, Hsu KW, Tsai JN et al (2005) Involvement of protein kinase C in the inhibition of lipopolysaccharide-induced nitric oxide production by thapsigargin in RAW 264.7 macrophages. Int J Biochem Cell Biol 37:2574–2585
29. Grove BD, Bruchey AK (2001) Intracellular distribution of gravin, a PKA and PKC binding protein, in vascular endothelial cells. J Vasc Res 38:163–175
30. Nelson PJ, Gelman IH (1997) Cell-cycle regulated expression and serine phosphorylation of the myristylated protein kinase C substrate, SSeCKS: correlation with culture confluency, cell cycle phase and serum response. Mol Cell Biochem 175:233–241