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Sparstolonin B suppresses lipopolysaccharide-induced inflammation in human umbilical vein endothelial cells

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Abstract

Sparstolonin B (SsnB) is an isocoumarin compound isolated from the tubers of both *Sparganium stoloniferum* and *Scirpus yagara*. We previously demonstrated that SsnB blocked the Toll-like receptor (TLR) 2- and TLR4-triggered inflammatory signaling in macrophages by inhibiting the recruitment of MyD88 to the TIR domains of TLR2 and TLR4. The present study was designed to examine the effects of SsnB on vascular inflammatory responses in human umbilical vein endothelial cells (HU-VECs) challenged by lipopolysaccharide (LPS, a TLR4 ligand). We found that SsnB dose-dependently attenuated the LPS-induced expression of interleukin (IL)-1 β and monocyte chemoattractant protein 1 both at the transcription and translation levels in HUVEC. LPS-induced endothelial cell adhesion molecules, intercellular adhesion molecular-1 and vascular cell adhesion molecule-1 expressions were also reduced by treatment with SsnB. In addition, co-

incubation with SsnB attenuated THP-1 monocyte adhesion to LPS-activated HUVECs. Furthermore, SsnB efficiently suppressed LPS-induced phosphorylation of extracellular-signal-regulated kinase (Erk1/2) and Akt in HUVECs. These findings show that SsnB can suppress endothelial cell inflammation, suggesting that SsnB might be suitable for development as a therapeutic agent for inflammatory cardiovascular disease.

Keywords

Sparstolonin B; Lipopolysaccharide; Endothelial cells; Inflammation; Cytokines; Monocyte adhesion

Introduction

The inflammatory responses of vascular endothelial cells to insults such as hyperlipidemia, infection and disturbed blood flow, play a pivotal role in the initiation and development of atherosclerosis, which is the primary cause of heart attack and stroke (Bains et al. 2010; Ross 1999; Fan and Watanabe 2003; Altman et al. 2008). When stimulated by pathogenic mediators, endothelial cells trigger critical inflammatory responses, including enhancement of endothelial permeability, secretion of cytokines/chemokines and recruitment of circulating leukocytes (Raetz and Whitfield 2002).

Lipopolysaccharide (LPS), the major portion of the outer membrane of Gram-negative bacteria, is an important pathogenic stimuli, and is a Toll-like receptor (TLR) 4 ligand (Wiedermann et al. 1999). LPS in the blood directly promotes vascular inflammation via the activation of resident cells such as monocytes and endothelial cells (Roth et al. 2007). It has been reported that endothelial cells, upon LPS stimulation, initiate the over-production of inflammatory cytokines such as interleukin (IL)-1 β , IL-8, TNF α , chemokines such as MCP-1, and adhesion molecules such as intercellular adhesion molecular-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Yamagami et al. 2003; Dauphinee and Karsan 2006).

Sparstolonin B (SsnB) is an isocoumarin compound, which is isolated from the tubers of both *Sparganium stoloniferum* and *Scirpus yagara* (Liang et al. 2011; Cui et al. 2012), and has long been used in the traditional Chinese medicine “SanLeng” for the treatment of several inflammatory diseases (Xi et al. 2002; Yang et al. 2010). Previously, we demonstrated that SsnB is a selective TLR2 and TLR4 antagonist (Liang et al. 2011). It blocks TLR2-and TLR4-triggered inflammatory signaling in macrophages by inhibiting the recruitment of MyD88 to TIR domains of TLR2 and TLR4. HUVECs, an endothelial cell line derived from human umbilical veins, are commonly used for in vitro experimental models of vascular endothelial cells. In this study, we investigated the effects of SsnB on the expression of LPS-induced inflammatory genes and adhesion molecules in HUVECs. Our results demonstrated that treatment of HUVECs with SsnB inhibited LPS-induced IL-1 β , MCP-1, ICAM-1 and VCAM-1 expression, decreased the adhesion of THP-1 monocytes to LPS-activated HUVECs, and attenuated LPS-induced phosphorylation of signaling molecules Erk1/2 and Akt.

Materials and methods

Chemicals and reagents

SsnB was isolated from the tubers of *S. stoloniferum* and *S. yagara* in our lab. The purity of the isolates were determined by HPLC to be >99 %, and their structure was determined by NMR spectroscopy and X-ray crystallography (Liang et al. 2011).

Primers were synthesized by Invitrogen (Carlsbad, CA). LPS (cell culture tested) was purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA). RPMI 1640, L-glutamine, streptomycin, and penicillin were purchased from Mediatech (Herndon, VA). Dulbecco's phosphate-buffered saline was purchased from HyClone (Logan, UT). HUVEC growth medium (F12K) was purchased from Sigma. Mouse monoclonal antibody to ICAM-1 was obtained from Zymed Laboratories Inc (San Francisco, CA). Rabbit anti-mouse VCAM-1 antibody was purchased from Santa Cruz (San Cruz, CA). Rabbit anti-Erk1/2 and anti-phospho-Erk1/2 antibodies were from Millipore (Billerica, MA). Rabbit anti-Akt and anti-phospho-Akt antibodies were from Cell Signaling Technology (Danvers, MA). Rabbit anti- β -actin antibody was from Sigma. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were from Millipore.

Cell culture

HUVEC cell line (CRL-1730) and the THP-1 human monocyte cell line (TIB-202™) were purchased from ATCC (Manassas, VA) both of which are immortal cell lines. HUVECs were grown in F12K medium containing 10 % FBS and growth supplements, such as heparin and endothelial cell growth supplement, and antibiotics. At confluence, the cells were subcultured at a 1:3 ratio, and were then cultured in a humidified chamber containing 5 % CO₂ at 37 °C. Cells at passage 2–6 were used for experiments. THP-1 cells were maintained in RPMI-1640 medium supplemented with 10 % FBS and appropriate antibiotics.

Quantitative real-time PCR (qPCR)

Total RNA was extracted and purified using TRIzol reagent (Invitrogen, San Diego, CA.) and RNeasy™ Mini kit (Qiagen, Germantown, MD) according to the manufacturers' instructions, and reverse transcription using a First-strand cDNA Synthesis System (Marligen Bioscience, MD) was then performed. Quantitative real-time PCR analyses was carried out using Fast Start Universal SYBR Green Master (Rox) (Roche, Indianapolis, Indiana) on an Eppendorf Realplex2 Mastercycler (Eppendorf, Hamburg, Germany). The primers used in qPCR were: human GAPDH (internal control), 5'-GGAGAAGGCTGGGGCTCAT-3' (forward) and 5'-TGATGGCATGGACTGTGGTC-3' (reverse); human MCP-1, 5'-CAGCCAGATGCAATCAATGCC-3'-(forward) and 5'-TGGAATCCTGAACCCACTTCT-3' (reverse); human IL-1 β , 5'-AGTTGCCTTCTTGGGACTGA-3' (forward) and 5'-TCCACGATTTCCAGAGAAC-3' (reverse). Samples were amplified using the following program: 95 °C for 10 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 15 s, and 68 °C for 20 s, followed by a melting curve analysis from 60 to 95 °C every 0.2 °C. The abundance of each gene product was

calculated by relative quantification, with values for the target genes normalized with GAPDH.

Enzyme-linked immunosorbent assay (ELISA)

The amounts of IL-1 β and MCP-1 in the culture medium were determined by ELISA, using the human IL-1 β and MCP-1 ELISA kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions. Supernatants of the HUVEC conditioned culture medium for IL-1 β and MCP-1 assays were diluted 60–120-fold prior to determinations, and the data were expressed relative to a standard curve prepared for IL-1 β and MCP-1.

Western blot analysis

Cell homogenates (40 μ g of protein) were separated by electrophoresis on 8–12 % SDS–polyacrylamide gels, and were transferred to a nitrocellulose membrane. The membranes were blocked with 5 % skim milk with 0.1 % Tween 20 (PBST) for 1 h, and were then incubated with primary antibodies overnight at 4 °C, including ICAM-1 (1:500), VACM-1 (1:500), anti-phospho (p)-Akt (1:1,000), anti-total (t)-Akt (1:1,000), anti-p-ERK(1:1,000), anti-t-ERK(1:1,000) and anti- β -actin (1:1,000). The membranes were then washed with PBST and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. After the final wash, protein bands were visualized using an ECL kit (Amersham, Arlington Heights, IL).

Monocyte cell adhesion assay

HUVECs were grown in F12k with 10 % FBS at a density of 1×10^5 cells/well in 24-well plates. After growth to confluence, cells were treated with both SsnB and LPS, alone and in combination, at the indicated concentrations in F12K with 1 % FBS for 20 h. THP-1 cells were labeled with RFP by lentiviral transduction as described previously (Fan et al. 2007). The labeled THP-1 cells were suspended in F12K medium with 1 % FBS, and were added to freshly washed HUVECs at 3×10^4 cells/well. Co-incubation was performed at 37 °C for 30 min and the non-adherent THP-1 cells were removed by gently by washing twice with PBS. The cells were then fixed for 30 min with 1 % glutaraldehyde. Fluorescent images were obtained using a Leica DMRX microscope (Wetzlar, Germany). The numbers of adherent RFP-expressing THP-1 cells were counted under a fluorescence microscope.

Statistical analysis

Results of quantitative experiments were expressed as mean values $\pm$ SE. Each data point represents the average of three independent experiments. One-way analysis of variance was used for multiple group comparison. When only two groups were compared, comparisons between groups were performed by Student's unpaired *t* test. Statistical significance was assigned to *p* values <0.05. GraphPad Prism 5 software was used to carry out all statistical analysis.

Results

SsnB decreases LPS-induced MCP-1 and IL-1 β mRNA expression in HUVECs

We have previously shown that SsnB at a concentration below 200 μ M did not cause observable cytotoxicity to HUVECs (Liang et al. 2011). Therefore we chose to use concentrations of 10 and 100 μ M SsnB for cell culture experiments throughout this study. We determined whether SsnB had inhibitory effects on the expression of inflammatory genes MCP-1 and IL-1 β in LPS-induced HUVECs by qPCR. As shown in Fig. 1a, incubation of HUVECs with 50 ng/ml of LPS for 6 h markedly increased the mRNA expression of both MCP-1 and IL-1 β . However, the increased MCP-1 mRNA level was dose-dependently blunted by the additional treatment of SsnB (10 μ M SsnB, 65.36 % of the LPS-treated group, $p < 0.05$; 100 μ M SsnB, 26.57 % of the LPS-treated group, $p < 0.01$); and IL-1 β mRNA level was also decreased in a dose-dependent manner when HUVECs were co-treated with SsnB (59.8 and 9.9 % of the LPS-treated group at 10 and 100 μ M SsnB, respectively, $p < 0.01$).

SsnB inhibits LPS-induced MCP-1 and IL-1 β protein secretion by HUVECs

To further confirm the inhibitory effects of SsnB on MCP-1 and IL-1 β expression in HUVECs, HUVECs were treated with SsnB and LPS for 16 h, and media were collected for measurement of cytokine concentrations using ELISA. As shown in Fig. 1b, in control groups, IL-1 β and MCP-1 levels in the media were almost undetectable. LPS (50 ng/ml) treatment dramatically increased the levels of both cytokines, whereas co-treatment with SsnB significantly reduced their levels in a dose-dependent manner. SsnB co-treatment decreased LPS-induced MCP-1 levels in the media to 64.7 and 30.6 % of the LPS-treated group at 10 and 100 μ M concentrations, respectively; and reduced LPS-induced IL-1 β to 64.8 and 13.3 % at these two concentrations, respectively.

SsnB suppresses LPS-induced expression of ICAM-1 and VCAM-1 in HUVECs

ICAM-1 and VCAM-1 are two of several adhesion molecules expressed by endothelial cells in response to several inflammatory stimuli, and are responsible for monocyte adhesion (Iiyama et al. 1999; Yoon et al. 2010). The effects of SsnB on the expression of ICAM-1 and VCAM-1 were tested using LPS-stimulated HUVECs by Western blot analysis (Fig. 2). The results demonstrated that LPS (50 ng/ml) significantly increased ICAM-1 and VCAM-1 expression in HUVECs by 2.5- and 50-fold, respectively. However, after the cells were co-treated with SsnB (10 or 100 μ M) for 24 h, the ICAM-1 and VCAM-1 levels induced by LPS were markedly reduced in a concentration-dependent manner. Specifically, SsnB reduced LPS-induced ICAM-1 expression by 17.9 and 43.8 % at 10 μ M and 100 μ M concentrations, respectively; while it diminished LPS-induced VCAM-1 expression ~10 and 25-fold at these two concentrations, respectively.

SsnB suppresses the adhesion of THP-1 cells to LPS-activated HUVECs

Monocyte recruitment to the endothelium is a crucial step in the inflammatory response which precedes the development of atherosclerosis. To determine whether the inhibition of MCP-1, IL-1 β , ICAM-1 and VCAM-1 expression by SsnB in LPS-induced HUVECs results

in a decrease in monocyte adhesion, we assessed THP-1 monocyte adhesion to HUVECs activated by LPS. Compared to non-treated HUVECs, the mono-layers of HU-VECs pre-treated with LPS (50 ng/mL) for 20 h attracted significantly more THP-1 cells for adhesion (Fig. 3). However, when HUVECs were co-treated with SsnB and LPS for 20 h, the number of adherent THP-1 cells was markedly reduced compared to those of the LPS-only group, in a SsnB dose-dependent manner (57.2 and 25.5 % of the LPS-only group at the SsnB concentration of 10 and 100 μ M, respectively, $p < 0.01$).

SsnB inhibits LPS-induced ERK1/2 and Akt phosphorylation in HUVECs

Next, we examined the effects of SsnB on the Erk1/2 MAPK signaling pathway, which is involved in the regulation of downstream proinflammatory gene expression in HUVECs (Hennig et al. 2006; Moon et al. 2011; Lin et al. 2005; Wang and Dong 2012; Libby 2002; Dauphinee and Karsan 2006). The results in Fig. 4 showed that LPS (50 ng/mL) treatment for 30 min induced a rapid increase in p-Erk1/2 protein levels in HUVECs. Co-treatment with SsnB at concentrations of 10 and 100 μ M reduced the level of p-Erk1/2 to 44.6 and 18.4 % that of the LPS-only group, respectively. Several studies have reported the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and its contribution to the up-regulated production of inflammatory cytokines in activated endothelial cells (Hennig et al. 2006; Moon et al. 2011). Thus, we also analyzed the effects of SsnB on LPS-induced PI3 K/Akt activation in HUVECs. As shown in Fig. 4, the level of p-Akt in HUVECs was rapidly increased after LPS exposure for 15 min. SsnB co-treatment resulted in the significant inhibition of LPS-induced Akt phosphorylation in a dose-dependent manner (45.4 and 11.8 % of that of the LPS-only group at SsnB concentration of 10 and 100 μ M, respectively).

Discussion

This work represents the continuation of our efforts to characterize SsnB as an anti-inflammatory agent. This study aimed to examine the anti-inflammatory effects of SsnB on vascular endothelial cells, using the HUVEC cell line as a model. SsnB is a 4'-phenyl isocoumarin isolated from ethanol extracts of the tubers of both *S. stoloniferum* and *S. yagara* (Liang et al. 2011; Cui et al. 2012). We recently reported the anti-inflammatory properties of SsnB in LPS-stimulated peritoneal macrophages and THP-1 monocytes (Liang et al. 2011). In this study, we demonstrated that SsnB also exerted anti-inflammatory effects on vascular endothelial cells.

Endothelial dysfunction, the initial step of atherogenesis, is closely related to the vascular inflammatory process, which seem to be the result of the interaction between exogenous stimuli and endothelial cells (Bains et al. 2010; Raetz and Whitfield 2002). One of the strongest stimulators that target the endothelium is LPS, which is both a unique glycolipid that makes up the outer membrane of Gram-negative bacteria, as well as a TLR4 ligand. Epidemiologic evidence has proven that circulating LPS constitutes a strong risk factor for the development of cardiovascular disease (Roth et al. 2007). In vivo and in vitro studies have also shown that high-level LPS exposure can trigger activation of endothelial cells, resulting in the secretion of proinflammatory cytokines, which further impact on

cardiovascular disease processes (Dauphinee and Karsan 2006). An important cytokine produced by ECs is MCP-1, which is the key player in monocyte recruitment, and high MCP-1 expression levels were determined in cases of cardiovascular disease (Libby 2002; Dauphinee and Karsan 2006). IL-1 β is another important cytokine which is expressed mainly within the endothelium of atherosclerotic plaques (Dewberry et al. 2000; Shemesh et al. 2011; Dengler et al. 2000). ICAM-1 and VCAM-1, produced by ECs, also act as key components in the inflammatory response, as well as in the recruitment of leukocytes to the sites of inflammation, and are thus implicated in the pathogenesis of vascular inflammatory diseases such as atherosclerosis (Fotis et al. 2012). In the present study, we demonstrated that stimulation with LPS (50 ng/ml) increased MCP-1, IL-1 β , ICAM-1 and VCAM-1 expression in HUVECs, while co-treatment with SsnB was able to dose-dependently suppress LPS-induced expression of all four.

The adhesion of monocytes to the endothelium is one of the earliest events in atherogenesis. This key step is mediated by an interaction between monocytes and the adhesion molecules such as VCAM-1 and ICAM-1, which are expressed on the endothelial cell surface (Iiyama et al. 1999). Since SsnB inhibited LPS-induced MCP-1, IL-1 β , ICAM-1 and VCAM-1 expression, we performed the monocyte adhesion assay. As expected, the results showed that co-incubation of HUVECs with SsnB significantly suppressed LPS-induced HUVEC and THP-1 interaction in a dose-dependent manner.

It has been reported that both the MAPK family and PI3 K/Akt signaling pathway are involved in the regulation of ICAM-1 and VCAM-1 expression (Hennig et al. 2006; Moon et al. 2011; Lin et al. 2005; Wang and Dong 2012; Libby 2002; Dauphinee and Karsan 2006). In addition, the activation of MAPKs has been shown to be associated with the expression of proinflammatory mediators which can trigger vascular inflammation (Hennig et al. 2006; Moon et al. 2011; Dauphinee and Karsan 2006; Liu et al. 2010). Thus, we investigated whether SsnB modulates Erk1/2 and Akt phosphorylation, which might contribute to the reduction of VCAM-1, ICAM-1, MCP-1 and IL-1 β expression in SsnB-treated HUVECs. Our results showed that the phosphorylation of Erk1/2 and Akt induced by LPS was dose-dependently decreased by SsnB in HUVECs. Since the activation of both Erk1/2 pathway and Akt pathway can be triggered by TLR4 signaling, we believe that the effects of SsnB on Erk1/2 and Akt phosphorylation in HUVECs are likely mediated by its inhibition of TLR4 signaling. We previously reported that SsnB blocked TLR2 and TLR4 signaling in mouse and human macrophages (Liang et al. 2011).

In conclusion, we have found that SsnB 1) effectively blocked LPS-induced expression of MCP-1, IL-1 β , ICAM-1, and VCAM-1 in HUVECs; 2) markedly decreased monocyte adhesion to LPS-activated HUVECs; and 3) significantly reduced LPS-induced phosphorylation of Erk1/2 and Akt in HUVECs. These findings suggest that SsnB might be suitable for development as an anti-inflammatory agent to suppress vascular inflammation and to prevent and treat cardiovascular diseases.

Acknowledgments

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Fig. 1.

Effects of SsnB on LPS-induced MCP-1 and IL-1 β expression in HUVECs. **a** HUVECs were treated with SsnB and LPS as indicated for 6 h. mRNA was extracted, and qPCR analysis was performed using specific primers for MCP-1, IL-1 β and GAPDH, as described in “Materials and Methods” Section. **b** HUVECs were treated with SsnB and LPS as indicated for 16 h. The levels of MCP-1 and IL-1 β in culture medium was measured by ELISA analysis. Data are expressed as mean values SE of three independent experiments, ^{##} $p < 0.01$ compared to the vehicle-treated group; ^{*} $p < 0.05$, ^{**} $p < 0.01$ compared to the LPS-treated group

Fig. 2. Effects of SsnB on the LPS-induced ICAM-1 and VCAM-1 expression in HUVECs. Cells were treated with SsnB and LPS as indicated for 24 h, were then lysed, and the protein levels were detected by Western blot analysis (*upper*). The blots were quantified by using densitometry, and represented by the ratio of β-actin (*lower*). Data are expressed as mean values SE of three independent experiments. ^{##} $p < 0.01$, versus vehicle-treated group; ^{*} $p < 0.05$, ^{**} $p < 0.01$ versus LPS-treated group

Fig. 3.

Effects of SsnB on monocyte adhesion to HUVECs. HUVECs were treated with SsnB and LPS as indicated for 20 h. Then the cells were co-incubated with fluorescence-labeled THP-1 cells for 30 min at 37 °C. After washing, adherent THP-1 cells were counted under a fluorescence microscope. Representative images were shown on the left (*a*, control; *b* LPS; *c* LPS + 10 μM SsnB; *d* LPS + 100 μM SsnB). The adherent THP-1 cell numbers were normalized to control group (*right*). Data are expressed as mean values SE of three independent experiments. ## $p < 0.01$ versus vehicle-treated group; * $p < 0.05$, ** $p < 0.01$ versus LPS-treated group

Fig. 4.

Effects of SsnB on phosphorylation of Erk1/2 and Akt induced by LPS in HUVECs. Cells were co-treated with LPS and different concentrations of SsnB, as indicated, for 30 min prior to detection of p-ERK1/2, or for 15 min prior to detection of p-Akt. Cells were extracted and protein levels were detected by Western blot analysis (*left*). Blots from three independent experiments were quantified by using densitometry and were normalized to control (*right*). ^{##} $p < 0.01$, versus vehicle-treated group; ^{**} $p < 0.01$ versus LPS-only group