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Saikosaponin C inhibits lipopolysaccharide-induced apoptosis by suppressing caspase-3 activation and subsequent degradation of focal adhesion kinase in human umbilical vein endothelial cells

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ABSTRACT

Bacterial lipopolysaccharide (LPS) is an important mediator of inflammation and a potent inducer of endothelial cell damage and apoptosis. In this study, we investigated the protective effects of saikosaponin C (SSc), one of the active ingredients produced by the traditional Chinese herb, *Radix Bupleuri*, against LPS-induced apoptosis in human umbilical endothelial cells (HUVECs). LPS triggered caspase-3 activation, which was found to be important in LPS-induced HUVEC apoptosis. Inhibition of caspase-3 also inhibited LPS-induced degradation of focal adhesion kinase (FAK), indicating that caspase-3 is important in LPS-mediated FAK degradation as well as in apoptosis in HUVECs. SSc significantly inhibited LPS-induced apoptotic cell death in HUVECs through the selective suppression of caspase-3. SSc was also shown to rescue LPS-induced FAK degradation and other cell adhesion signals. Furthermore, the protective effects of SSc against LPS-induced apoptosis were abolished upon pretreatment with a FAK inhibitor, highlighting the importance of FAK in SSc activity. Taken together, these results show that SSc efficiently inhibited LPS-induced apoptotic cell death via inhibition of caspase-3 activation and caspase-3-mediated-FAK degradation. Therefore, SSc represents a promising therapeutic candidate for the treatment of vascular endothelial cell injury and cellular dysfunction.

1. Introduction

The integrity of the endothelial lining is essential for maintaining vascular homeostasis and normal organ function. Disruption of this lining, either by injury or through cellular dysfunction, has been implicated in the pathogenesis of numerous vascular diseases, such as atherosclerosis and sepsis. Endothelial cell apoptosis can be a risk factor for vascular diseases, resulting in a loss of endothelial integrity followed by vascular leakage, inflammation, and coagulation. Lipopolysaccharide (LPS), an endotoxic component of the cell wall of gram-negative bacteria, is a potent inducer of innate immunity in mammalian systems. Exposure to LPS leads to the release of numerous pro-inflammatory cytokines, thereby accelerating the inflammatory process. Indeed, LPS-mediated inflammation has been implicated in the pathogenesis of much of the endothelial cell injury and dysfunction which are associated with vascular diseases [1–5]. LPS induces apoptosis in different types of endothelium [2,3,6–8]. LPS-mediated apoptosis also plays a major role in disease pathogenesis via induction of apoptotic mediators, such as caspase-1 and caspase-3 [8–10].

The survival of endothelial cells is dependent upon interactions with multiple components of the extracellular matrix mediated through focal adhesions; disruption of these integrin-mediated cell–matrix interactions has been shown to induce apoptosis [11]. Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that plays a critical role in transmitting signals from the extracellular matrix to the cytoplasm, and it is an important component of the focal adhesion complex. Disruption of FAK signaling results in apoptosis via caspase-mediated proteolysis of focal adhesions [12,13], and overexpression of FAK can prevent apoptosis [14–16].

The saikosaponins are an important class of structurally related compounds derived from the traditional Chinese herbal *Radix Bupleuri*. These compounds have been shown to be effective for the treatment of a variety of conditions, including fever, inflammation-associated influenza, hepatic injury, and the common cold. Among the saikosaponins produced by *Radix Bupleuri*, saikosaponins A, B1/B2, C, and D are the most abundant. Saikosaponins A, B, and D have been shown to inhibit growth in cancer cells via induction of apoptosis [17–20]. Saikosaponin C (SSc), however,

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exhibits no effect on cell growth [20,21]; this difference is likely due to structural differences between SSc and other saikosaponins. Instead, SSc has been found to induce endothelial cell growth, migration, and capillary tube formation, thereby contributing to angiogenesis [20].

This study aimed to investigate the effects of SSc on LPS-induced endothelial cell apoptosis. We found that SSc inhibited LPS-induced apoptotic cell death in HUVECs. This effect was mediated via inhibition of caspase-3 activation and subsequent FAK degradation, suggesting a mechanism by which SSc is able to protect against LPS-mediated apoptosis.

2. Materials and methods

2.1. Reagents

LPS (from *Escherichia coli* O111:B4), MG132, and 3-methyladenine were obtained from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO). SSc was obtained from Nacalai Tesque (Kyoto, Japan). The caspase-3 inhibitor z-DEVD-fmk and FAK inhibitor were purchased from Calbiochem (La Jolla, CA).

2.2. Cell culturing

HUVECs were obtained from Cambrex (Walkersville, MD, USA) at passage 1. Cells were routinely maintained on 1% gelatin-coated plates in M199 medium with 20% FBS, bovine endothelial cell growth supplement (30 ng/ml), and heparin (90 ng/ml), and grown at 37 °C in a humidified 5% CO_2 environment. HUVECs at passage 3–8 were used.

2.3. SSc treatment

A stock solution (15 mM) of SSc was prepared in DMSO and stored at −20 °C until use. The concentration of DMSO, <0.2% (vol/vol), used in the present study both as a vehicle for SSc and as a control, had no damaging effect on the HUVEC cells. HUVECs were incubated with various concentrations (5–30 μM) of SSc for 2 h before treatment with 1 μg/ml LPS.

2.4. Treatments with various inhibitors

For the caspase-3 activity assay, apoptosis assay and FAK degradation measurement, HUVECs were pretreated with caspase-3 inhibitor z-DEVD-fmk (20 μM) or FAK inhibitor (20 nM) for 2 h, and then exposed to 1 μg/ml LPS in the presence or absence of SSc (30 μM) pretreatment. Sometimes, HUVECs were pretreated with proteasome inhibitor MG132 (5 μM) or autophagy inhibitor 3-methyladenine (5 mM) for 2 h and then treated with 1 μg/ml LPS.

2.5. Cell death assay

Following 1 μg/ml LPS treatment for 40 h in the presence or absence of a variety of concentrations of SSc, the rate of HUVEC cell death was measured using the Trypan blue exclusion assay. Viable cells were determined based on their ability to exclude the dye.

2.6. Flow cytometric detection of the sub-G1 fraction

Following 1 μg/ml LPS treatment for 30 h in the presence or absence of a variety of concentrations of SSc, cellular DNA was stained with propidium iodide (PI) and quantified by sub-G1 fluorescence-activated cell sorter (FACS) analysis using the FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). After fixation with cold 70% ethanol, the cells were washed with DPBS twice and stained with DPBS containing PI (50 μg/ml; Sigma) and RNase A (50 μg/ml; Sigma). The cell suspension was incubated in the dark at room temperature for 30 min, and 10,000 cells were measured per sample. The analysis was performed using the Cell Quest Pro software (Becton Dickinson).

2.7. Flow cytometric detection of phosphatidylserine expression

Cells were harvested at 24 h after treatment with 1 μg/ml LPS in the presence or absence of a variety of concentrations of SSc and stained with PI and annexin-V (Becton Dickinson) for 15 min at room temperature then analyzed using flow cytometry (Becton Dickinson). PI and annexin V emissions were detected in the FL-2 and FL-1 channels, respectively. Data from 10,000 cells were recorded in list mode on logarithmic scales. Data analysis was conducted using Cell Quest Pro software (BD Biosciences). Annexin V-positive cells were defined as apoptotic cells.

2.8. Caspase-3 activity assay

Cells were treated with 1 μg/ml LPS in the presence or absence of caspase-3 inhibitor z-DEVD-fmk (20 μM) or FAK inhibitor (20 nM) and/or a variety of concentrations of SSc for 20 h. Caspase colorimetric assay kit (BioVision, Palo Alto, CA) specific for caspase-3 was used to determine the enzymatic activities of caspase-3 based on assaying the cleavage of a synthetic colorimetric substrate. Cell lysates were normalized for protein content and incubated with the reaction buffer and labeled substrates at 37 °C for 2 h. Substrate for caspase-3 activity assay was DEVD-*p*-nitroanilide (DEVD-pNA). Caspase-3 activity was measured by spectrophotometric detection of the chromophore pNA at 405 nm after it was cleaved from the substrate DEVD-pNA.

2.9. Immunoblot analysis

Immunoblotting was performed to monitor apoptotic signaling and cell adhesion signaling. HUVEC cells were harvested at 20 h after treatment with 1 μg/ml LPS in the presence or absence of various concentrations of inhibitors and/or SSc. Cell were solubilized using lysis buffer [50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM EGTA, 1% Triton X-100, 1 mM DTT] containing protease inhibitor cocktail and phosphatase inhibitor cocktail. Protein samples (10 μg/lane) were separated by SDS–PAGE and transferred onto PVDF membranes (NEN, PerkinElmer, Wellesley, MA). The blots were probed with specific primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. Signals were detected using the ECL or ECL-Plus chemiluminescence system (Amersham Biosciences).

2.10. Statistical analysis

Statistical analyses were performed using the SAS 8.1 software for Windows (SAS Institute, Inc., Cary, NC). All experiments were repeated three times and the results are presented as means ± standard error. Data were evaluated using the one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison *post-hoc* test, and differences were considered significant at $P < 0.05$.

3. Results

3.1. Inhibitory effects of SSc on LPS-induced apoptosis in HUVECs

LPS confers cytotoxic effects in endothelial cells through induction of apoptosis [2,3,6]. In contrast, SSc dramatically improved

both the viability and growth of endothelial cells [20], with no cytotoxic effects observed for up to 48 h (Fig. 1A). Our primary interest was therefore to determine whether SSc could inhibit LPS-induced cytotoxicity and apoptosis.

Using a trypan blue exclusion assay, pretreatment of HUVECs with SSc was shown to inhibit LPS-stimulated death in a dose-dependent manner (Fig. 1B). Next, flow cytometry was used to examine the rate of apoptosis in treated cells via detection of sub-G1 DNA content and using the annexin V binding assay. LPS-treated HUVEC cell populations contained higher numbers of sub-G1 cells (Fig. 1C) and annexin V-positive cells (Fig. 1D), compared with untreated controls. Pretreatment with SSc effectively abolished this effect, with both sub-G1 and annexin V-positive cell numbers similar to that of controls.

3.2. Critical role of caspase-3 in LPS-induced apoptosis in HUVECs

LPS is known to induce caspase activation in endothelial cells [3,8–10]. To examine the role of different caspases in LPS-induced apoptosis, HUVECs were pre-incubated with selective caspases inhibitors prior to exposure to LPS and then analyzed to determine the rate of apoptosis and caspase activation. As shown in Fig. 2A, LPS activated caspase-3; other caspases, such as caspase-8 and caspase-9, were not activated by LPS treatment (data not shown). Selective inhibition of caspase-3 could be achieved using the z-DEVD-fmk inhibitor. Selective inhibition of caspase-3 effectively suppressed the LPS-induced increase in the sub-G1 cell fractions (Fig. 2B, left panel) and the loss of membrane integrity (Fig. 2B, right panel), indicating that caspase-3 specifically is important in LPS-induced apoptosis in HUVECs.

3.3. Critical role of caspase-3 in LPS-induced FAK degradation in HUVECs

FAK plays an important role in the maintenance of focal adhesions and cytoskeletal structures in many cells [22]. FAK exhibits anti-apoptotic properties in an anchorage-dependent manner [14,16] and is a target for degradation by caspase-3 [13], resulting in apoptosis. LPS-induced FAK degradation was observed following treatment with LPS (Fig. 2C), consistent with previous results [8]. This degradation was efficiently abolished by pretreatment with inhibitor z-DEVD-fmk, indicating a role for caspase-3 in LPS-mediated FAK degradation. However, LPS-induced FAK degradation was not blocked by either MG132 (an inhibitor of proteasome) or 3-methyladenine (an inhibitor of autophagy) (Fig. 2D), suggesting that neither 26S proteasome pathway nor autophagic/lysosomal degradation pathway was involved in LPS-induced FAK degradation. Several studies have also reported direct effect of caspases on FAK degradation [12,13].

3.4. Inhibitory effects of SSc on LPS-induced activation of caspase-3

To examine the effects of SSc on LPS-induced caspase-3 activation, cells were pre-incubated with SSc before exposure to LPS; caspase-3 activation was determined using immunoblotting to examine the cleavage of caspase-3 and PARP, and by using

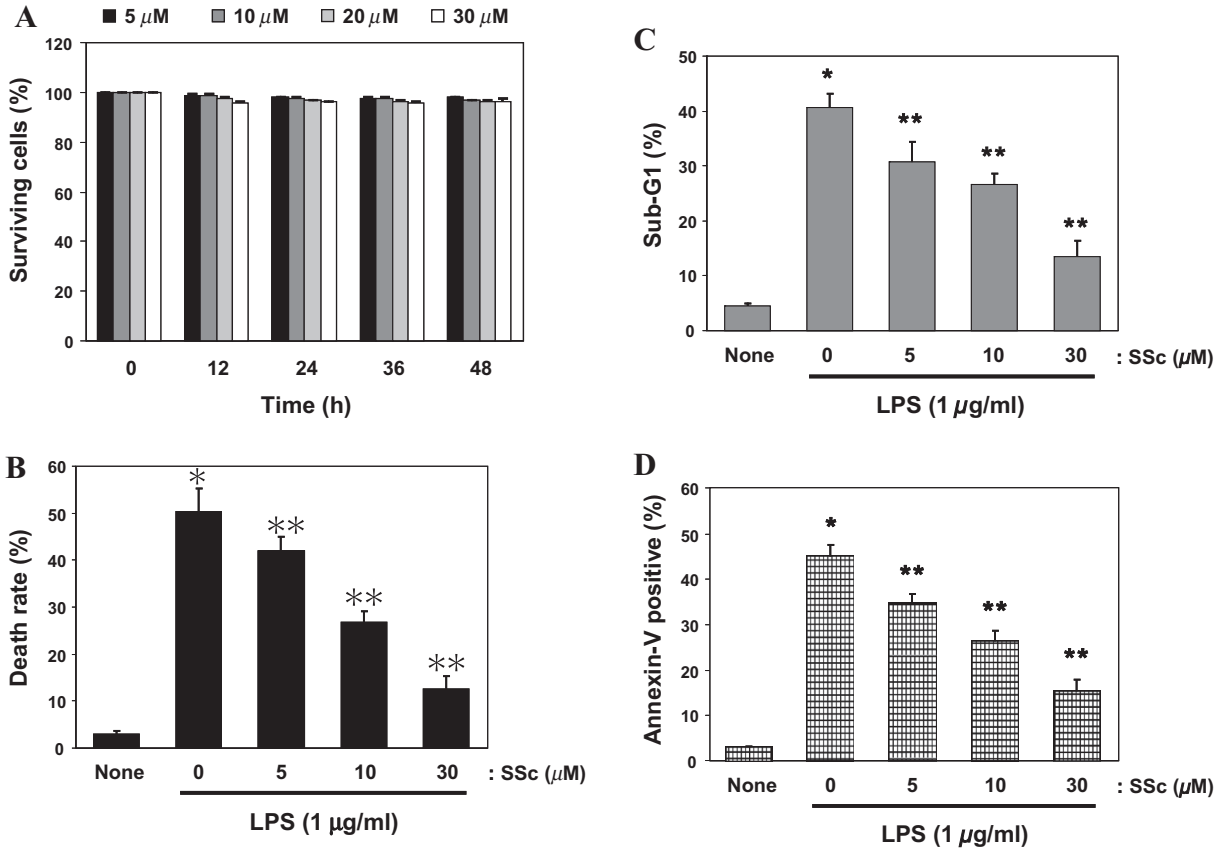

Fig. 1. SSc inhibits LPS-induced apoptosis in human endothelial cells. (A) HUVECs were treated with the indicated concentrations of SSc for up to 48 h, and the cell viabilities were measured. (B–D) HUVECs were challenged with 1 μg/mL LPS for 40 h (for trypan blue staining), 30 h (for measurement of sub-G1 DNA content), or 24 h (for annexin V staining) in the presence or absence of different SSc concentrations as indicated. The percentage of trypan blue (B), sub-G1 (C), and annexin V-positive (D) cells were determined and compared among treatment groups. Each column represents the mean ± standard error of three independent experiments (*$P < 0.05$ vs. untreated controls; **$P < 0.05$ vs. LPS alone; ANOVA/Dunnett's test).

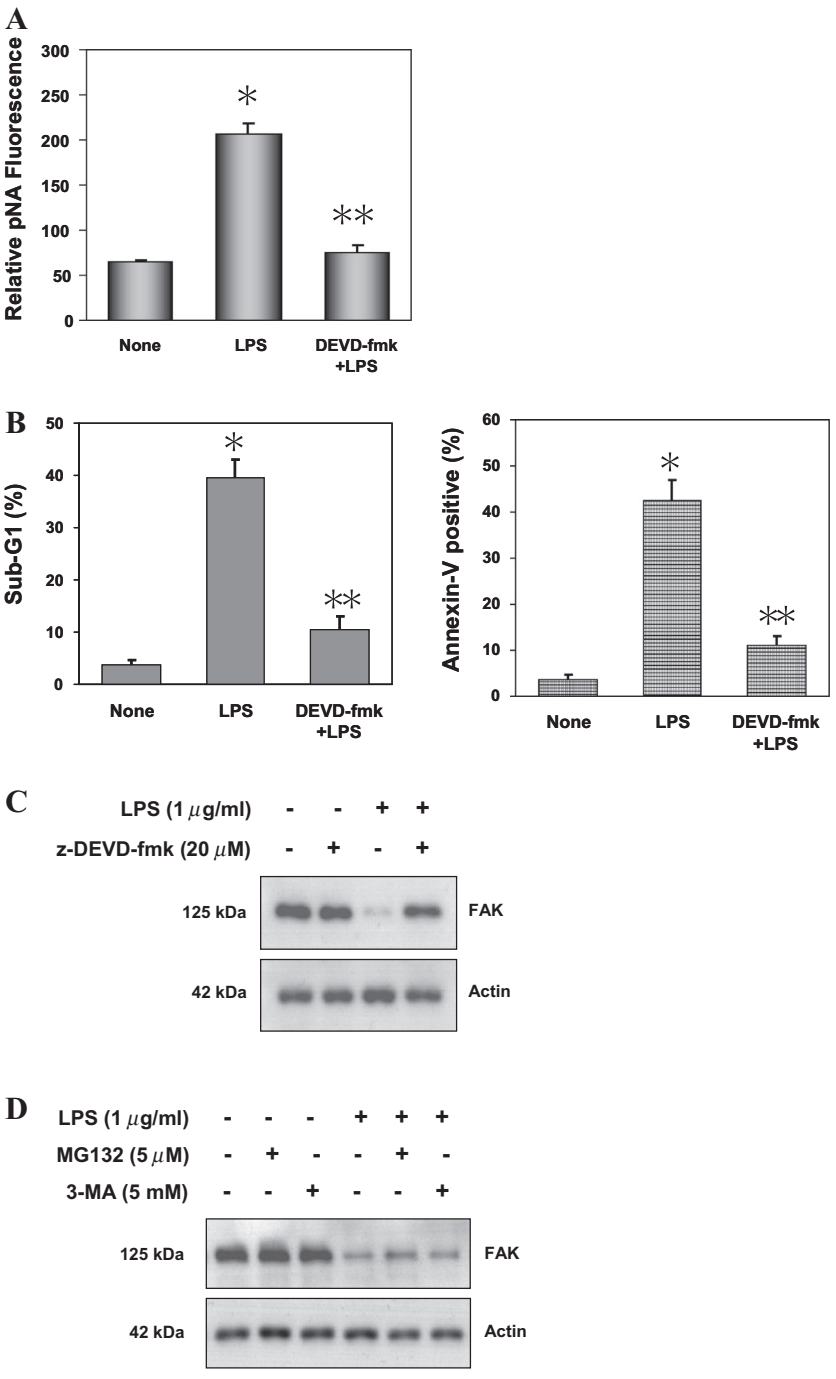

Fig. 2. Caspase-3 is involved in LPS-induced apoptosis and FAK degradation. HUVECs were challenged with 1 μg/mL LPS for 30 h (for measurement of sub-G1 DNA content), 24 h (for annexin V staining), or 20 h (for measurement of caspase-3 activity and protein expression) in the presence or absence of 20 μM caspase-3 inhibitor z-DEVD-fmk or 5 μM proteasome inhibitor MG132 or 5 mM autophagy inhibitor 3-methyladenine (3-MA). (A) Changes in caspase-3 activity were monitored by detection of pNA liberated from the DEVD-pNA substrate. All samples were measured in triplicate and each data point represents the mean ± standard error of three independent experiments (*$P < 0.05$ vs. untreated controls; **$P < 0.05$ vs. LPS alone; ANOVA/Dunnett's test). (B) Apoptotic cells were quantified using flow cytometry with PI staining of the sub-G1 population (left panel) and annexin V staining (right panel). Each column represents the mean ± standard error of three independent experiments (*$P < 0.05$ vs. untreated controls; **$P < 0.05$ vs. LPS alone; ANOVA/Dunnett's test). (C and D) Expression levels of FAK and actin were estimated using immunoblot analysis with specific antibodies. The blots are representative of three independent experiments.

colorimetric caspase-3 activity assay. Pre-incubation with SSc significantly attenuated LPS-induced cleavage of both caspase-3 and PARP, and LPS-induced caspase-3 activation, in a dose-dependent manner (Fig. 3A and B). These results indicate that inhibition of caspase-3 is important for the preventative effects of SSc on LPS-induced apoptosis in HUVECs.

3.5. Inhibitory effects of SSc on LPS-induced FAK degradation

Given the involvement of FAK degradation in LPS-induced apoptosis [8], we next sought to determine whether SSc can also inhibit LPS-mediated FAK degradation. Pre-incubation with SSc significantly inhibited LPS-mediated FAK degradation (Fig. 4A).

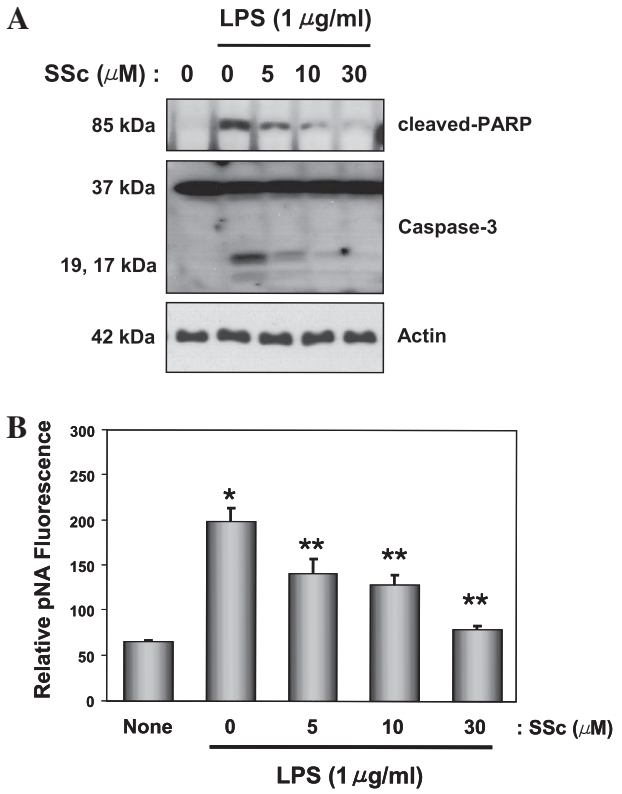

Fig. 3. SSc suppresses LPS-induced caspase-3 activation. HUVECs were challenged with 1 μg/mL LPS for 20 h in the presence or absence of the indicated concentrations of SSc. (A) Western blotting was used to detect caspase-3 and cleaved PARP. The blots are representative of three separate experiments. (B) Changes in the activity of caspase-3 were monitored via the detection of pNA liberated from the DEVD-pNA substrate. All samples were measured in triplicate and each data point represents the mean ± standard error of three independent experiments (*$P < 0.05$ vs. untreated controls; **$P < 0.05$ vs. LPS alone; ANOVA/Dunnett's test).

FAK functions upstream of PI3K/Akt to transduce a beta integrin survival signal [23]. LPS treatment resulted in a loss of Akt kinase activity, as determined by Ser473 phosphorylation status, and this decrease in activity was effectively abolished following pretreatment with SSc (Fig. 4A). Interestingly, LPS significantly downregulated the activity of molecules associated with cell–cell adhesions, including VE-Cadherin and p38; this downregulation was significantly inhibited by SSc pretreatment (Fig. 4A). These results indicate that the loss of endothelial cell-specific adhesion molecules may be implicated in LPS-induced cytotoxic apoptosis in endothelial cells. Treatment with SSc effectively restored the activity of these adhesion molecules, thereby inhibiting apoptotic cell death.

3.6. SSc attenuation of LPS-induced apoptosis is mediated by inhibition of FAK degradation

To examine the role of FAK on SSc activity, we used a selective FAK inhibitor. Although the FAK inhibitor is known to inhibit FAK catalytic kinase activity (IC_{50} = 4 nM), it did not affect LPS-induced change in FAK expression in the presence or absence of SSc (Fig. 4B). SSc inhibited LPS-induced apoptosis, as evidenced by decreases in the sub-G1 cell population and in annexin V staining; these cytoprotective effects were significantly reduced following pre-treatment with a pharmacological inhibitor of FAK (Fig. 4C). siRNA inhibition of FAK also inhibited the protective effects of SSc in HUVECs (data not shown). Despite the evident role of FAK in SSc activity, FAK inhibition did not affect the suppressive action of SSc against LPS-induced caspase-3 activation (Fig. 4D). Taken together, these results indicate that SSc exerts its protective effects on LPS-induced HUVEC apoptosis through the inhibition of caspase-3-mediated FAK degradation induced by LPS.

4. Discussion

Inflammation plays a key role in the pathology of many vascular conditions, including atherosclerosis and sepsis. Septic responses to inflammatory signals such as LPS, have been shown to disrupt endothelial barrier function both *in vitro* and *in vivo* [1–4], driven in part by increased apoptosis in endothelial cells [2,3,6]. As apoptosis of endothelial cells is a contributing factor in many of these conditions, its suppression might be a useful for the treatment of vascular endothelial diseases. However, the mechanisms underlying LPS-induced apoptosis are still unclear. The data presented here clearly demonstrate a role for caspase-3-mediated FAK degradation in LPS-induced apoptosis of endothelial cells. Pretreatment of cells with SSc, one of the active compounds derived from the traditional Chinese herb *Radix Bupleuri*, significantly attenuated these effects in HUVECs, suggesting a potential role for this compound in the treatment of vascular diseases.

First, SSc was shown to significantly inhibit LPS-induced apoptotic cell death in HUVECs. The concentrations of SSc used in this study exhibited no cytotoxic effects on any of the cell types tested (data not shown), marking an important difference between SSc and other saikosaponins [17–21]. Additionally, lactate dehydrogenase, a soluble cytosolic enzyme, was released into the culture medium following LPS treatment. This release was significantly inhibited by SSc pretreatment, indicating that LPS-treated cells also undergo late apoptosis or secondary (post-apoptotic) necrosis, as well as typical apoptosis, which is inhibited by SSc pretreatment (data not shown).

Caspases, a family of cysteine proteases, are the central regulators of apoptosis. LPS has been shown to activate a number of caspases in multiple cell types [3,8–10,24]. Here, we found that caspase-3 was activated after LPS treatment, while other caspases, such as caspase-8 and caspase-9, were unaffected. The role of caspase-3 was analyzed using a selective caspase-3 inhibitor. This analysis revealed that caspase-3 was important in LPS-induced HUVEC apoptosis. Furthermore, inhibition of caspase-3 significantly abolished LPS-induced FAK degradation, indicating that FAK cleavage is induced by caspase-3 during LPS-induced apoptosis in endothelial cells. Pretreatment of cells with SSc significantly inhibited LPS-induced activation of caspase-3, confirming the role of caspase-3 in LPS-induced apoptosis. Our results showed that LPS also activated caspase-1 but caspase-1 is not important in LPS-induced apoptosis and FAK degradation in HUVECs (data not shown). These findings were in line with the results that pre-incubation with SSc had no effect on LPS-induced cleavage and activation of caspase-1 (data not shown). Since caspase-1 plays an important role in inflammation and provides a link between inflammation and apoptosis [8,9,25], our results indicate that LPS-induced apoptosis and FAK degradation are not relevant to LPS-induced pro-inflammatory responses.

Endothelial cells undergo apoptosis when cell adhesion and spreading are prevented [13,26], implying a requirement for anti-apoptotic signals from the extracellular matrix for cell survival. FAK, a non-receptor tyrosine kinase, acts as an early modulator of the integrin signaling cascade, regulating basic cellular functions, such as motility, proliferation, and survival [22]. Given that FAK has also been shown to exert anti-apoptotic effects [14–16], its degradation might be an important event in endothelial cell apoptosis. LPS-mediated HUVEC apoptosis was shown to involve FAK degradation [8]; this degradation was significantly abolished following pretreatment with SSc. LPS was also shown to inhibit the phosphorylation and activation of Akt, VE-Cadherin, and p38,

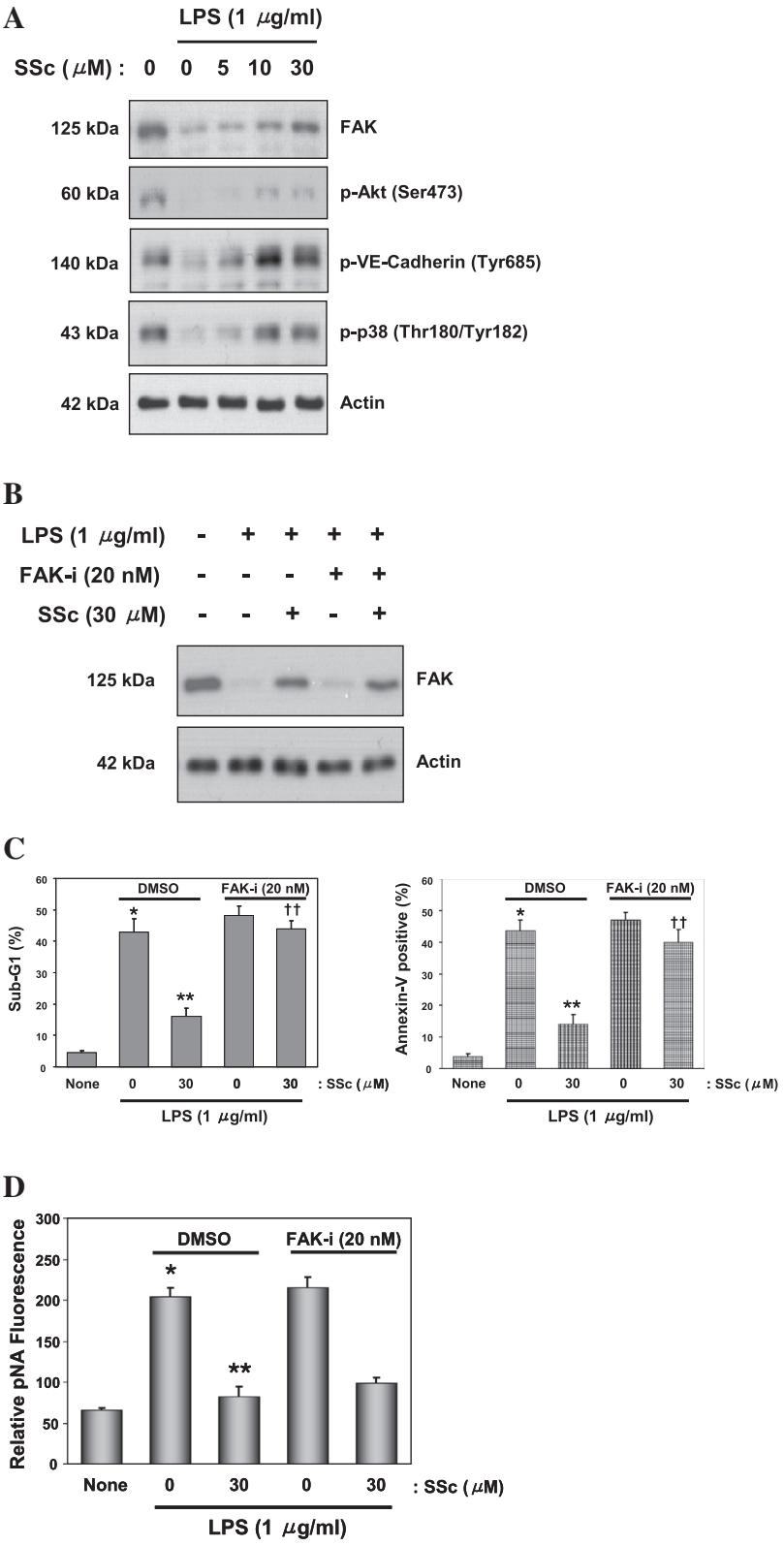

Fig. 4. SSc attenuation of LPS-induced apoptosis is mediated by inhibition of FAK degradation. (A) HUVECs were challenged with 1 μg/mL LPS for 20 h in the presence or absence of the indicated concentrations of SSc. FAK, phospho-Akt, phospho-VE-Cadherin, and phospho-p38 levels were assessed using total and phospho-specific antibodies. The blots shown are representative of three independent experiments. (B–D) HUVECs were pretreated with a FAK inhibitor (20 nM; FAK-i) or vehicle control for 2 h, followed by treatment with SSc (30 μM) or vehicle control for an additional 2 h. Cells were then challenged with 1 μg/mL LPS for 30 h (for measurement of sub-G1 DNA content), 24 h (for annexin V staining), or 20 h (for measurement of caspase-3 activity and protein expression) in the presence or absence of the FAK inhibitor and/or SSc. (B) Expression levels of FAK and actin were estimated using immunoblot analysis with specific antibodies. The blots are representative of three independent experiments. (C) Apoptosis was determined by sub-G1 population quantification (left panel) and annexin V staining (right panel). Each column represents the mean ± standard error of three independent experiments ($^{*}P < 0.05$ vs. untreated controls; $^{**}P < 0.05$ vs. LPS alone; $^{\dagger\dagger}P < 0.05$ vs. LPS in the presence of 30 μM SSc; ANOVA/Dunnett's test). (D) Changes in caspase-3 activity were monitored by the detection of liberated pNA. Each column represents the mean ± standard error of three independent experiments ($^{*}P < 0.05$ vs. untreated controls; $^{**}P < 0.05$ vs. LPS alone; ANOVA/Dunnett's test).

which were protected by SSc pretreatment. Interestingly, all of these proteins have been implicated in vascular endothelial growth factor (VEGF) signaling. Because VEGF also inhibits LPS-induced apoptosis [8], the association between VEGF and SSc may be worth investigating.

Finally, we showed that FAK degradation was the key factor mediating the protective effects of SSc in LPS-induced HUVEC apoptosis. Inhibition of FAK by pharmacological inhibition or by siRNA knockdown markedly abolished the protective effects of SSc against LPS-induced HUVEC apoptosis, confirming the essential role of FAK degradation in LPS-induced apoptosis. However, inhibition of FAK did not affect the SSc-mediated inhibition of LPS-induced caspase-3 activation. Together, these results suggest that FAK degradation is the crucial downstream effect of caspase-3 activation during LPS-induced apoptosis.

Taken together, our results demonstrate that LPS-induced apoptosis of HUVECs involves FAK degradation, as well as activation of caspase-3, and that the sequential activation of caspase-3, leading to FAK degradation, is essential for LPS-induced apoptosis, which was effectively abolished by SSc pretreatment. Our data also suggest that SSc represents a novel potential therapeutic for the prevention of vascular injury which is associated with endothelial apoptosis. Further investigation into the possible role of SSc against endothelial cell apoptosis and injury induced by stimuli other than LPS is needed.

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