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Study on the mechanisms of an extract of *Salvia miltiorrhiza* on the regulation of permeability of endothelial cells exposed to tumour necrosis factor-α

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Abstract

Exposure of endothelial cells to tumour necrosis factor-α (TNF-α) results in increased endothelial permeability, accompanied by a loss of cell–cell adherence junctions. The importance of tyrosine phosphatase and kinase activity in oxidant-mediated loss of cell junction structures has been demonstrated. The purpose of this study was to determine whether tyrosine phosphorylation contributes to TNF-α-mediated disorganization of endothelial cell junctions and how an extract of *Salvia miltiorrhiza* (ESM) and its active ingredients, Danshensu (DSS) and salvianolic acid B (Sal B), exert their protective effect in maintaining cell integrity. Immunoblotting results indicated that TNF-α exposure resulted in tyrosine phosphorylation of junctional proteins such as vascular endothelial cadherin and β-catenin, which was attenuated by ESM and its active ingredients DSS and Sal B. In addition, immunoprecipitation showed ESM and its active ingredients prevented β-catenin disassociation from the cytoskeleton in TNF-α-treated human umbilical vein endothelial cells. The results suggest that TNF-α produced biological effects at least partly by junctional protein phosphotyrosine modifications by increasing the total cellular phosphorylation level. It could be concluded that ESM and its active ingredients were effective at eliminating the factors leading to the rise in cellular phosphorylation, thus helping to maintain the integrity of endothelial junction structure.

Introduction

The junctional structures between endothelial cells are critical for maintaining the barrier between circulating components of blood and subendothelial tissues. Similar to epithelial cells, endothelial cells have specialized junctional regions called adherens junctions and tight junctions. In addition to the well known role of tight junctions in maintaining the blood–brain barrier (Rubin & Staddon 1999), the literature highlights the adherens junction as a major component in the pathophysiological regulation of paracellular permeability of the microvascular endothelium. The endothelial adherens junction complex is primarily comprised of vascular endothelial cadherins (VE-cadherins) and catenins, including α-catenin and β-catenin, connected to the actin cytoskeleton (Lampugnani et al 1995). VE-cadherins form Ca^{2+}-dependent homotypic bonds with other cadherins on opposed cells, resulting in adherens junction formation (Takeichi 1988; Siflinger-Birnboim & Malik 1996).

Many environmental factors can damage the structure and function of endothelial cells, leading to disease. One such factors is tumour necrosis factor-α (TNF-α), a pro-inflammatory cytokine that is released in response to pathological conditions. TNF-α can injure the structure of endothelial cells and increase endothelial permeability through the formation of inter-endothelial gaps and redistribution of junctional proteins (Lum & Malik 1994; Dudek & Garcia 2001; Ding et al 2005a). The pathways that regulate VE-cadherin-mediated cell–cell adhesion within endothelial cells are poorly defined. Cellular contractility has been proposed to be important, while others have reported an important role for tyrosine phosphorylation of the VE-cadherin complex as a means of regulating cell–cell adhesion in endothelial cells (Esser et al 1998; Braga et al 1999; Wetering et al 2002).

Salvia miltiorrhiza, a Chinese medicinal herb, has long been used for the treatment of atherosclerosis. It was recently reported to be effective in the amelioration of CCl_4-mediated

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hepatic apoptosis in rats (Lee et al 2006), and the induction of cytochrome P450-dependent monooxygenase in mice (Kuo et al 2006). Our previous study demonstrated that TNF-α exposure resulted in the loss of in-vitro endothelial barrier properties and that an extract of *S. miltiorrhiza* (ESM), in particular its active constituents, Danshensu (DSS) and salvianolic acid B (Sal B), significantly inhibited the TNF-α-induced increase in endothelial permeability by inhibiting vascular endothelial growth factor (VEGF) expression and subsequent gap formation (Ding et al 2005a). However, the molecular targets of ESM and its active constituents involved in their signal transduction pathways remain largely unknown. The purpose of this study was to determine the mechanisms involved in ESM-mediated inhibition of increased permeability of endothelial cells exposed to TNF-α. We investigated whether ESM and its active constituents mediated their effects on maintaining cell integrity through regulation of tyrosine phosphorylation of the VE-cadherin complex.

Materials and Methods

Materials

Sal B and protocatechualdehyde (purity >98%) were purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). DSS (purity >99%) was obtained from the College of Pharmacy in Fudan University (Shanghai, China). Endothelial cell growth medium was purchased from Clonetics (San Diego, CA, USA). Fetal bovine serum was purchased from HyClone (Logan, Utah, USA). Trypsin/EDTA, HEPES, PMSF, penicillin, streptomycin, gelatin, collagenase type II, bovine serum albumin (BSA), DAB and *o*-phenylene diamine dihydrochloride (OPD) were obtained from Sigma (St Louis, MO, USA). Recombinant human TNF-α was obtained from Pepro Tech EC Ltd (London, UK). Antibodies for VE-cadherin, β-catenin, phosphorylated-tyr204 (PY99), HRP-linked secondary antibody and ECL reagent kit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals used were of the highest grade commercially available.

Preparation of ESM

ESM was provided by the Research Institute of Tianjin Tasly Group Co. Ltd (Tianjin, China). The extract was prepared as previously described (Ding et al 2005b). For analysis of the constituents of ESM, the aqueous extract was dissolved in water and filtered through a 0.2-μm polyvinylidene fluoride filter. Reverse-phase high performance liquid chromatography (HPLC) analysis was performed using an HPLC system (HP1100; Agilent Technologies, Inc., Santa Clara, CA, USA). The constituents of the extracts were separated by a C18 column (5 μm, 4.6×250 mm) with elution gradients of 3% v/v (10 min), 10% v/v (10 min) and 20% v/v (20 min) CH_3CN in water/acetate acid (25:1) at a flow rate of 0.5 mL min^{-1}. The result confirmed the presence of the major active constituents in the aqueous extract of *S. miltiorrhiza*. DSS, Sal B and protocatechualdehyde were detected at 280 nm and quantified using purified products as standards.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from freshly obtained human umbilical cords by collagenase type II treatment as previously described (Stangl et al 2001). The cells were cultured in gelatin-coated culture flasks containing an endothelial growth medium supplemented with 10% fetal bovine serum, 100 U mL^{-1} penicillin, 100 μg m L^{-1} streptomycin, at 37°C in a humidified environment containing 5% CO_2. The endothelial cells were identified by its typical "cobblestone" morphology, and staining of Von Willebrand factor by immunohistochemisty. The purity of HUVECs in cultures was greater than 95%, and passages from 1 to 3 were used in this study.

Phosphotyrosine measurements

The phosphotyrosine content of HUVECs was measured by the method described by Kevil et al (2001) with slight modification. Briefly, HUVECs were cultured to confluency in 96-well plates. Cells were then incubated with 200 and 400 μg mL^{-1} ESM, 10 and 20 μg mL^{-1} DSS, and 10 and 20 μg mL^{-1} Sal B for 12 h, followed by TNF-α (100 ng mL^{-1}) for 6 h. Monolayers were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at 4°C, permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature and blocked with 2% BSA in PBS at 4°C overnight. Primary antibody for phosphorylated-tyr204 (PY99) (1:200) in 0.1% BSA, and PBS solution was added to the wells for 1 h at room temperature. Monolayers were washed three times with the 0.1% BSA and PBS solution. Horseradish peroxidase-conjugated secondary antibody of goat anti-mouse IgG (1:5000) in 0.1% BSA, and PBS solution were then added to wells for 1 h at room temperature. Wells were developed using peroxidase substrate OPD, and the reaction was terminated with 2 M H_2SO_4. The absorbance of each well was measured at 492 nm using a microplate reader. For the negative control group, primary antibody was substituted with PBS. The value of absorbance of the sample minus that of the negative control group was used as a measurement of phosphotyrosine content, normalized to cells incubated in control medium, which were considered 100%.

Cellular phosphatase activity

HUVECs were cultured as described above using 96-well plates. Treatment protocols were the same as used for phosphotyrosine measurements. After treatment, cells were incubated with 50 mg mL^{-1} *p*-nitrophenylphosphate for 30 min. Reactions were terminated using 2 M NaOH and absorbance was read using a plate reader at 492 nm.

Immunofluorescence

Confluent cells were treated with media alone, 400 μg mL^{-1} ESM, 20 μg mL^{-1} Sal B and 20 μg mL^{-1} DSS for 12 h before incubation with TNF-α at 100 ng mL^{-1} for 6 h. Cells were then fixed with 2% paraformaldehyde at 4°C for 30 min, permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature and treated with a blocking buffer (10%

goat serum) at room temperature for 30 min. These fixed cells were further incubated with a solution containing a specific mouse anti-phosphorylated-tyr204 antibody for 3 h at room temperature, followed by incubation with FITC-conjugated secondary antibody of goat anti-mouse IgG for 1 h. The plates were mounted and examined by Nikon fluorescence microscopy (Eclipse, Tokyo, Japan); images were captured and analysed by a Spot advanced digital camera and software.

Immunoprecipitation

Confluent cells were treated with media alone, 400 μg mL^{-1} ESM, 20 μg mL^{-1} Sal B and 20 μg mL^{-1} DSS for 12 h before incubation with TNF-α at 100 ng mL^{-1} for 6 h. Cells were washed with cold PBS and collected by centrifugation at 900 *g* for 1 min at 4°C. They were lysed by suspension in lysis buffer (0.5 M NaCl, 50 mM Tris, 1 mM EDTA, 0.05% SDS, 0.5% TritonX-100 and 1 mM PMSF) for 30 min at 0°C, followed by centrifugation at 23 000 *g* for 30 min at 4°C. For all experimental groups, equal amounts of protein from the cell-lysed extracts were analysed. Each sample was made up to 1 mL with lysis buffer and rotated for 3 h at 4°C with excessive anti-VE-cadherin or anti-β-catenin antibody, and incubated with protein-A agarose at 4°C overnight. The immunoprecipitated pellets were collected by centrifugation at 20 000 *g* for 10 min and washed three times with PBS. Pellets were resuspended in sample buffer and boiled for 3 min. Equal supernatants were applied to 10% SDS-PAGE for separation. Separated proteins were transferred to a nitrocellulose membrane that was subsequently blocked with 5% skim milk/TBS-Tween 20 (0.05%) at 4°C overnight. The treated membrane was incubated for 2 h at room temperature with a specific mouse anti-phosphotyrosine antibody, followed by washing, incubation for 1 h at room temperature with HRP-conjugated goat anti-mouse IgG secondary antibody, and signal detection by exposure to a Kodak film via treatment with a chemiluminescent reagent. VE-cadherin and β-catenin expression was used as an internal standard for each sample. Specificity of these primary antibodies was confirmed by an experiment in which primary antibodies were left out.

Cytoskeletal association assay

Cytoskeletal association assay of HUVECs was conducted according to the method described by Esser et al (1998). HUVECs were grown to confluency in 60-mm dishes. Treatment protocols were the same as used for the immunoprecipitation experiment above. After treatment, confluent HUVEC cultures were washed twice with PBS, followed by a 3-min extraction with 0.5% Triton X-100 in TBS. The extraction buffer was collected, centrifuged and the supernatant defined as the Triton-soluble fraction. Following extraction, the cells appeared to be homogeneously adherent to the culture vessel, with well-preserved nuclei and cytoskeletal fibres as judged by phase contrast microscopy. The Triton-insoluble components were then extracted with 0.5% SDS and 1% NP-40 in TBS for 20 min on ice. The extract was collected, vigorously pipetted, centrifuged and the supernatant was used as the cytoskeletal-associated fraction.

Statistical analysis

The results for each group were expressed as mean ± s.e.m. The effect of ESM on each parameter was examined using the one-way analysis of variance followed by the Student–Newman–Keuls multiple range test. Significance was determined at $P < 0.05$.

Results

Analysis of ESM by HPLC

Using HPLC analysis, DSS, Sal B and protocatechualdehyde were detected as the major constituents of ESM; the content of the three ingredients in ESM was 0.72 ± 0.02%, 0.43 ± 0.01% and 0.49 ± 0.02%, respectively (Figure 1).

Effect of ESM on phosphotyrosine content and phosphatase activity in TNF-α-treated HUVECs

To investigate the mechanism of the protective effect of ESM and its major ingredients, tyrosine phosphorylation levels were studied by enzyme-linked immunosorbent assay under various conditions. As expected, TNF-α induced a significant increase in tyrosine phosphorylation (Table 1). Compared with the control group incubated in media alone, 100 ng mL^{-1} TNF-α increased tyrosine phosphorylation by about 4-fold after 6 h treatment. It seems that the TNF-α-induced increase in permeability is associated with an increase in tyrosine phosphorylation. To determine if treatment with ESM inhibits the elevation of tyrosine phosphorylation, we pretreated

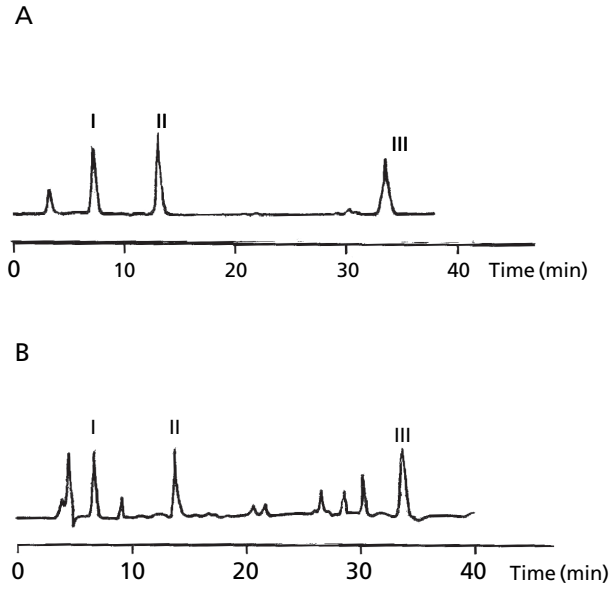

Figure 1 A. HPLC chromatogram of Danshensu (DSS), protocatechualdehyde and salvianolic acid B (Sal B). B. HPLC chromatogram of the extract of *Salvia miltiorrhiza*. I, DSS; II, protocatechualdehyde; III, Sal B.

Table 1 Effects of an extract of *Salvia miltiorrhiza* (ESM) and its active constituents, Danshensu (DSS) and salvianolic acid B (Sal B), on phosphotyrosine content and phosphatase activity in human umbilical vein endothelial cells (HUVECs) exposed to tumour necrosis factor-α (TNF-α)

	Phosphotyrosine content	Phosphatase activity
Control	0.233 ± 0.03	0.915 ± 0.14
TNF-α	0.965 ± 0.08	0.310 ± 0.10
TNF-α + ESM (200 μg mL^{-1})	0.563 ± 0.07*	0.551 ± 0.12*
TNF-α + ESM (400 μg mL^{-1})	0.475 ± 0.05*	0.643 ± 0.09*
TNF-α + DSS (10 μg mL^{-1})	0.615 ± 0.06*	0.613 ± 0.10*
TNF-α + DSS (20 μg mL^{-1})	0.457 ± 0.04*	0.762 ± 0.08*
TNF-α + Sal B (10 μg mL^{-1})	0.685 ± 0.05*	0.543 ± 0.12*
TNF-α + Sal B (20 μg mL^{-1})	0.524 ± 0.05*	0.674 ± 0.07*

HUVECs were treated with various concentrations of ESM, DSS and Sal B. Data represent the mean ± s.e.m., n = 4. *$P < 0.05$ compared with the TNF-α alone group.

HUVEC monolayers with various concentrations of ESM for 12 h before TNF-α stimulation. As shown in Table 1, a dose-dependent reduction in tyrosine phosphorylation was observed. About 50% reduction in tyrosine phosphorylation was achieved by ESM at 400 μg mL^{-1}. The effects of the major constituents of ESM, namely DSS and Sal B, were also studied. DSS at 10 and 20 μg mL^{-1} had the greatest activity, in agreement with its protective effect on permeability (Ding et al 2005a). Sal B at 10 and 20 μg mL^{-1} also inhibited tyrosine phosphorylation but to a lesser extent.

Table 1 shows the level of cellular phosphatase activity in various conditions. TNF-α (100 ng mL^{-1}) caused about 70% reduction in phosphatase activity compared with the control group after 6 h treatment. Pretreatment of HUVEC monolayers with various concentrations of ESM, DSS and Sal B for 12 h prevented the decrease in phosphatase activity. A 1.1-fold increase in phosphatase activity was achieved by ESM at 400 μg mL^{-1} compared with the TNF-α alone group. DSS at 20 μg mL^{-1} elevated phosphatase activity by 2.5-fold, and Sal B at 20 μg mL^{-1} elevated phosphatase activity by 2.2-fold. The data in Table 1 clearly shows the correlation between tyrosine phosphorylation level and phosphatase activity. The results suggest that the inhibitory effect of ESM and its major ingredients DSS and Sal B on cellular tyrosine phosphorylation might be attributed to their ability to prevent the decrease in phosphatase activity in TNF-α-treated HUVECs.

Effect of ESM on phosphotyrosine at intercellular contacts in TNF-α-treated HUVECs

Since decreased cell–cell adhesion and increased permeability of epithelial and endothelial cell monolayers has been associated with elevated tyrosine phosphorylation levels of junction components (Kinch et al 1995; Staddon et al 1995; Lampugnani et al 1997), we investigated if ESM and its active components might reduce the increased permeability in TNF-α-treated HUVECs by regulating the level of phosphotyrosine at intercellular contacts. The phosphotyrosine signal in unstimulated HUVEC monolayers was very diffuse and showed only weak staining at focal adhesions (Figure 2A). TNF-α stimulated the phorsphotyrosine labelling, which was

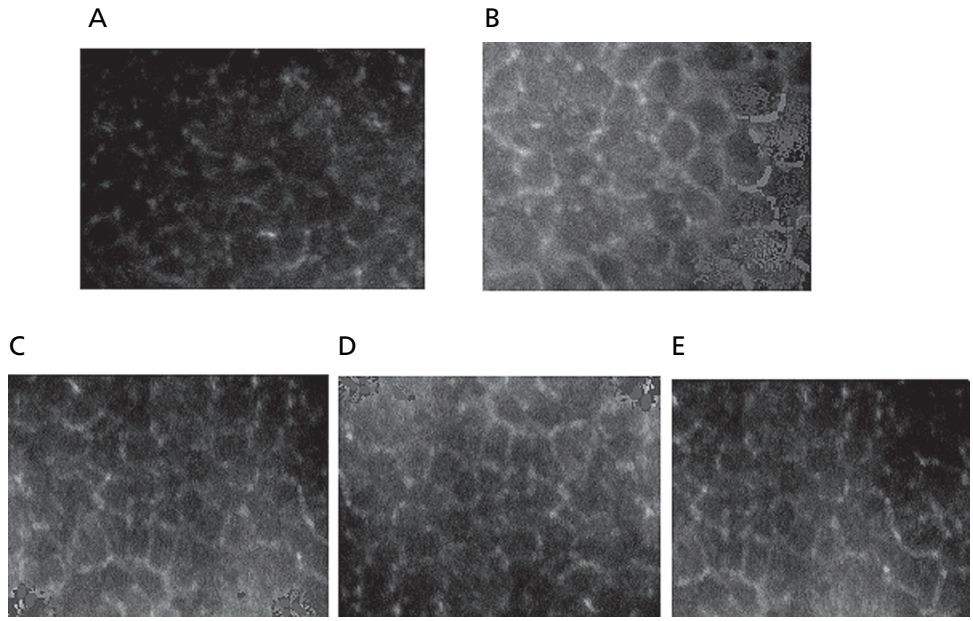

Figure 2 Phosphotyrosine at intercellular contacts. Human umbilical vein endothelial cells (HUVECs) were pretreated with the extract of *Salvia miltiorrhiza* (ESM), Danshensu (DSS) and salvianolic acid B (Sal B) for 12 h before 6 h incubation with tumour necrosis factor-α (TNF-α; 100 ng mL^{-1}). After removing TNF-α, the cells were treated with the anti-phosphorylated-tyr204 antibody and then stained with FITC-conjugated secondary antibody. Phosphotyrosine at intercellular contacts was analysed using a fluorescence microscope (magnification ×100). A. Unstimulated HUVECs. B. TNF-α stimulated HUVECs. C–E. HUVECs pretreated with ESM (400 μg mL^{-1}), DSS (20 μg mL^{-1}) and Sal B (20 μg mL^{-1}), respectively.

already evident after 30 min (Figure 2B). The increase in the immunofluorescence staining of focal adhesion was markedly reduced by pretreatment with ESM at 400 μg mL^{-1} (Figure 2C), DSS at 20 μg mL^{-1} (Figure 2D) and Sal B at 20 μg mL^{-1} (Figure 2E).

Effect of ESM on tyrosine phosphorylation of junctional proteins in TNF-α-treated HUVECs

To determine whether TNF-α induced alteration of the phosphotyrosine signal at the intercellular junctions by increasing the tyrosine phosphorylation of components of the cell–cell adhesion junction complex, we conducted the immunoprecipitation of cell adhesion molecules with anti-phosphotyrosine antibody. We focused on two constituents of the adherens junction complex, VE-cadherin and β-catenin, in endothelial cells. After 6 h of treatment, TNF-α induced significant tyrosine phosporylation of the two proteins, as evidenced by intensive bands on the Western blots. When HUVECs were pretreated with ESM at 400 μg mL^{-1}, DSS at 20 μg mL^{-1} and Sal B at 20 μg mL^{-1}, the tyrosine phosporylation of VE-cadherin and β-catenin was attenuated to varying degrees (Figure 3), which coincided with the tyrosine phosphorylation

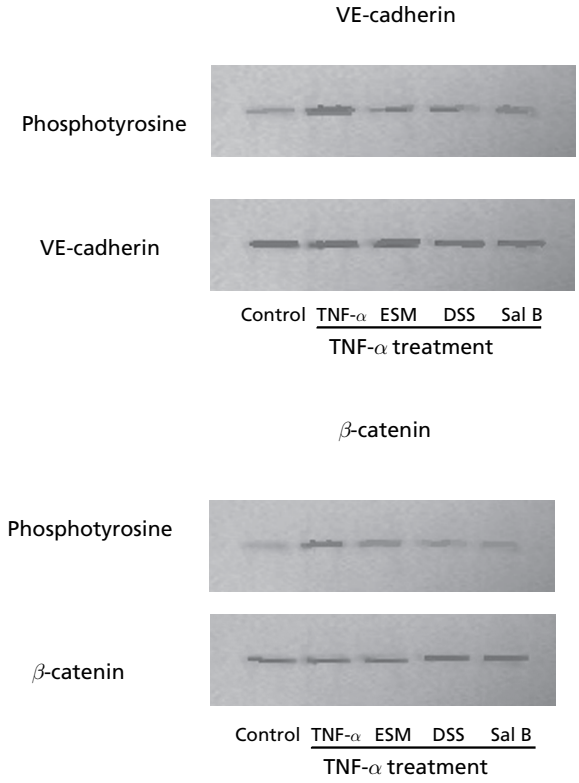

Figure 3 Tyrosine phosphorylation of the vascular endothelial cadherin (VE-cadherin) complex. Human umbilical vein endothelial cells were pretreated with the extract of *Salvia miltiorrhiza* (ESM; 400 μg mL^{-1}), Danshensu (DSS; 20 μg mL^{-1}) and salvianolic acid B (Sal B; 20 μg mL^{-1}) for 12 h before incubation with tumour necrosis factor-α (TNF-α; 100 ng mL^{-1}) for 6 h. After removing TNF-α, tyrosine-phosphorylated VE-cadherin and β-catenin were determined by immunoprecipitation analysis.

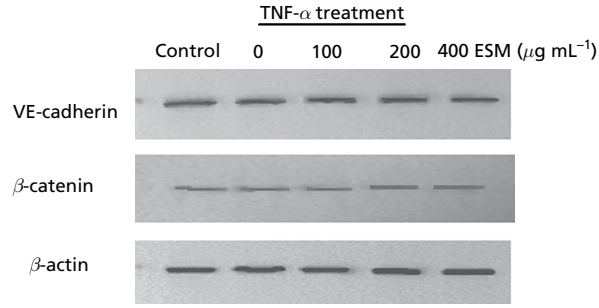

Figure 4 Protein expression. Human umbilical vein endothelial cells were pretreated with the extract of *Salvia miltiorrhiza* (ESM; 100, 200 and 400 μg mL^{-1}) for 12 h before incubation with tumour necrosis factor-α (TNF-α; 100 ng mL^{-1}) for 6 h. After removing TNF-α, vascular endothelial cadherin (VE-cadherin) and β-catenin expression were determined by Western blot analysis.

at cell–cell contacts, as shown in Figure 2. In addition to the phosphorylated modification of junctional proteins, we questioned whether the increase in endothelial permeability involves decreased protein expression of the two major components of the junction structure. As shown in Figure 4, we found no statistically significant differences in the quantity of VE-cadherin and β-catenin in the presence of TNF-α alone or ESM, DSS and Sal B plus TNF-α. These results suggest that the alteration of endothelial permeability by TNF-α is not regulated at the translational level but involves tyrosine phosporylation of junctional proteins, and that ESM and its active constituents mediate their effects through the tyrosine phosporylation signal pathway.

Effect of ESM on association of β-catenin with the cytoskeleton in TNF-α-treated HUVECs

In order to determine whether the association of β-catenin with the actin cytoskeleton changes with the VE-cadherin complex being phosphorylated, the amount of β-catenin contained in the Triton X-100-insoluble fraction was determined by Western blotting, Triton X-100 insolublility being indicative of a molecule associated with the cytoskeleton. A considerably decreased amount of β-catenin in the Triton X-100-insoluble fraction, through detergent extraction, was observed upon TNF-α stimulation, indicating that TNF-α induced dissociation of β-catenin from the cytoskeleton, which was inhibited to varying degrees by pretreatment with ESM at 400 μg mL^{-1}, DSS at 20 μg mL^{-1} and Sal B at 20 μg mL^{-1} (Figure 5). These results suggest the correlation between phosphorylation of the VE-cadherin complex and dissociation of β-catenin from the cytoskeleton.

Discussion

It has previously been reported that H_2O_2 at concentrations that mediate an increase in permeability can cause an increase in tyrosine kinase activity and a decrease in phosphatase activity (Kevil et al 2001; Nwariaku et al 2004).

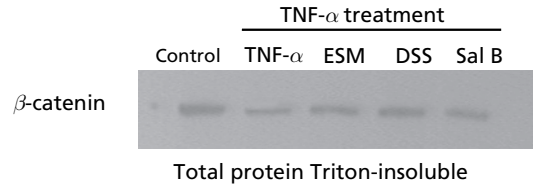

Figure 5 Association of β-catenin with cytoskeleton. Human umbilical vein endothelial cells were pretreated with the extract of *Salvia miltiorrhiza* (ESM; 400 μg mL^{-1}), Danshensu (DSS; 20 μg mL^{-1}) and salvianolic acid B (Sal B; 20 μg mL^{-1}) for 12 h before incubation with tumour necrosis factor-α (TNF-α; 100 ng mL^{-1}) for 6 h. After removing TNF-α, the association of β-catenin with cytoskeleton was determined through detergent extraction and Western blot analysis.

This agrees with our results that phosphotyrosine content was increased and phosphatase activity was reduced in TNF-α-treated HUVECs (Table 1). H_2O_2 activated pp60 Src kinase and other members of the Src kinase family, which led to phosphorylation of adhesion proteins and subsequent hyperpermeability of endothelial cells through the decrease in the adhesive force of interendothelial cells (Lee et al 2004). Another crucial factor contributing to the increased phosphotyrosine content might be the decreased activity of phosphatase. Kevil et al (2001) demonstrated that phosphatase activity was more sensitive to reactive oxygen species (ROS) exposure. They agreed that critical cysteine residues responsible for protein tyrosine phosphatase activity are specifically oxidized in response to ROS (Zick & Sagi-Eisenberg 1990; Denu & Tanner 1998), and therefore phosphatase activity declined. It has been proved that ROS activates cell phosphotyrosine pathways. Our previous study showed that TNF-α stimulation induced an increase in the intracellular ROS level, while pre-incubation of HUVECs with ESM and its active constituents, DSS and Sal B, eliminated intracellular ROS, and increased the glutathione level. This might contribute to enhancing the activity of phosphatase by lowering its oxidation, therefore inhibiting the TNF-α-induced increase in phosphotyrosine content in HUVECs.

A previous study showed that TNF-α exposure markedly induced overexpression of VEGF in endothelial cells (Ding et al 2005a). VEGF and its receptors constitute an important regulatory system, regulating growth and differentiation of the cells. Overexpression of VEGF activates expression of VEGF receptor-2 (Esser et al 1998), which is a type of transmembrane tyrosine kinase and catalyses phosphotyrosine of cell proteins. Effectors that are ignited by VEGF receptor-2 also include Src protein kinase and mitogen-activated protein kinase (D'Angelo et al 1995; Ukropec et al 2000; Vorbrodt & Dobrogowska 2003). These kinases may also facilitate the cell protein phosphotyrosine in direct or indirect ways. In this experimental system, the ability of ESM, DSS and Sal B to prevent the TNF-α-induced cell protein phosphotyrosine might be attributable to their inhibitory effect on VEGF overexpression. We have previously demonstrated that TNF-α caused formation of intracellular gaps and cell rounding, however no obvious changes were observed in VE-cadherin and β-catenin expression levels in TNF-α-treated endothelial cells with or without pretreatment of various concentrations of ESM (see Figure 4). This suggests that the disturbance of endothelial junctional protein distribution associated with increased endothelial permeability is not regulated at the translational level, but might involve some type of post-translational control, as suggested by previous studies (Ukropec et al 2002; Zhang et al 2003). The pathways that regulate VE-cadherin-mediated intercellular adhesion are largely unknown. An important role for tyrosine phosphorylation of the VE-cadherin complex as a means to regulate cell–cell adhesion in endothelial cells has been reported (Esser et al 1998; Braga et al 1999; Wetering et al 2002). The results in Table 1, Figure 2 and Figure 3 show that the phosphotyrosine of VE-cadherin and β-catenin of HUVECs could be observed following TNF-α stimulation, indicating that junctional proteins become phosphorylated. However, in accordance with a reduction in the phosphotyrosine content by ESM and its active constituents, they also showed the ability to block the phosphotyrosine of VE-cadherin and its signalling partner β-catenin.

Phosphotyrosine of junctional proteins has been proved to be closely associated with cell integrity (Chou et al 2002; Weis et al 2004). Lampugnani et al (1995) showed that endothelial cadherin associations with the actin cytoskeleton are dependent on β-catenin linkage. In the present study, the results showed that β-catenin associated with actin cytoskeleton was significantly decreased in TNF-α-treated HUVECs, while in HUVECs pretreated with ESM, DSS and Sal B, its decrease was inhibited to varying degrees, indicating that ESM and its active ingredients prevented disassociation of junctional proteins from the cytoskeleton. Tyrosine phosphorylation promotes the dissociation of junctional proteins from their cytoskeletal anchors (Rao et al 2002). Many agonists that are classified as hyperpermeability factors stimulate β-catenin phosphorylation and simultaneously cause endothelial gap formation (Wong et al 1999; Tinsley et al 2002). The inhibition of ESM and its active constituents on hyerpermeability of TNF-α-treated HUVECs might be due to their blocking the phosphorylation of the VE-cadherin complex, including β-catenin. This builds a foundation for their subsequent prevention of the disassociation of β-catenin from the cytoskeleton.

Conclusion

This study demonstrated that ESM and its major constituents, DSS and Sal B, significantly inhibited TNF-α-induced increase in tyrosine phosphorylation in HUVECs. The results also showed that these ingredients effectively attenuated the phosphorylation of junction proteins, namely VE-cadherin and its signal partner β-catenin in TNF-α-treated HUVECs, and the disassociation of β-catenin from the cytoskeleton, which is crucial for cell integrity. Because of the key roles of the structure and function of vascular endothelial cells in inflammation and cardiovascular dysfunction, this research on cultured HUVECs provides useful insights into the pharmacological efficacy of *S. miltiorrhiza*.

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