TSLP/TSLPR promote angiogenesis following ischemic stroke via activation of the PI3K/AKT pathway

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Abstract. The current study aimed to investigate the effects of the thymic stromal lymphopoietin (TSLP)/TSLP receptor (TSLPR) on angiogenesis following ischemic stroke *in vivo* and *in vitro*. Furthermore, whether the phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT) pathway mediates the effects of TSLP/TSLPR on angiogenesis was explored. A rat middle cerebral artery occlusion (MCAO) model was established, and it was demonstrated that the expression levels of TSLP and TSLPR were significantly increased in the infarct area between 12 and 72 h after MCAO, as determined by ELISA and western blot analyses. TSLP injection was revealed to upregulate vascular endothelial growth factor A (VEGFA) and angiopoietin 2 (Ang-2) expression levels in the infarct area following MCAO, as determined by western blot analysis. An *in vitro* MCAO model was constructed by exposing human umbilical vein endothelial cells (HUVECs) to oxygen-glucose deprivation (OGD). It was revealed that the expression levels of TSLP and TSLPR were significantly increased in HUVECs subjected to OGD. TSLP treatment was revealed to induce *in vitro* angiogenesis by promoting cell proliferation and migration, and increasing tube length of OGD-treated HUVECs, as determined by MTT, Transwell-migration and tube formation assays, respectively. Furthermore, it was demonstrated that the PI3K/AKT pathway was activated by TSLP treatment. However, it was revealed that PI3K inhibitor, LY294002, could attenuate the effects of TSLP on *in vitro* angiogenesis of OGD-treated HUVECs. In conclusion, to the best of our knowledge, this study demonstrated for the first time that TSLP/TSLPR promote angiogenesis following ischemic stroke *in vivo* and *in vitro*. Furthermore, it was demonstrated that the effects of TSLP/TSLPR on angiogenesis were, at least partially, mediated via activation of the PI3K/AKT pathway. TSLP/TSLPR may serve as a potential therapeutic target for ischemic stroke treatment.

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Introduction

Stroke is a life-threatening condition with a high mortality rate and a high risk of subsequent disability (1,2). Ischemic stroke is the most common form of stroke, accounting for ~85% of the total number of strokes (3). Despite advances in current stroke therapies, many patients do not benefit from conventional treatments.

Ischemic strokes are caused by a blockage of the arteries responsible for the provision of blood to the brain or spinal cord, therefore resulting in critically reduced blood flow to said region(s). Well-functioning collateral circulation has been demonstrated to improve the clinical prognosis following an ischemic stroke (4-6). Angiogenesis is the generation of new blood vessels from pre-existing vasculature (7). A series of studies have revealed that post-ischemic angiogenesis contributes to the improvement in neurological functional recovery following a stroke (8). Therefore, angiogenesis has been suggested to be a promising therapeutic target for ischemic stroke.

Thymic stromal lymphopoietin (TSLP), a member of the interleukin 7 cytokine family, is predominantly produced by epithelial cells, fibroblasts and smooth muscle cells (9,10). TSLP signals via a TSLP receptor (TSLPR), which is widely distributed among a number of different immune cells, such as mast cells, monocytes, dendritic cells and lymphocytes (11-16). Thus, TSLP has been suggested to be involved in the modulation of both innate and adaptive immune responses (17-20). Previously, Xie *et al* (21) reported that TSLP is able to modulate the biological behavior of vascular endothelial cells *in vitro*, and is also involved in the angiogenesis of cervical cancer. However, the exact role of TSLP/TSLPR in angiogenesis following ischemic stroke has not previously been investigated.

In the present study, the biological role of TSLP/TSLPR in angiogenesis in rats subjected to middle cerebral artery occlusion (MCAO), and human umbilical vein endothelial cells (HUVECs) subjected to oxygen-glucose deprivation (OGD) were investigated. Furthermore, whether or not the phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT) pathway can mediate the effects of TSLP/TSLPR on angiogenesis following ischemic stroke was determined.

Materials and methods

Animals. This study was approved by the Ethics Committee of Hunan Provincial People's Hospital (Changsha, China), and all of the experiments performed on animals were performed in compliance with the principles of experimental animal ethics. A total of 48 Sprague-Dawley male rats at the age of 8 weeks, weighing 300-350 g, were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The rats were maintained under controlled conditions (22±2˚C; 55% humidity) with a 12 h light/dark cycle, and free access to food and fresh water. The permanent middle cerebral artery occlusion (MCAO) model was established in accordance with the Longa *et al* study (22). Briefly, the rats in the MCAO group were anesthetized via an intraperitoneal injection of 10% chloral hydrate (300 mg/kg intraperitoneal injection). Subsequently, the right common carotid artery was exposed through a 2 cm midline incision in the neck. To occlude the middle cerebral artery, a 4-0 nylon suture with a silicone tip was inserted into the internal carotid artery until mild resistance was felt. At 6, 12, 24 and 72 h time intervals following MCAO, the rats were sacrificed via an intraperitoneal injection of pentobarbital (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Rats in the sham group were anesthetized and underwent surgery without MCAO. The neurological function of the rats was then tested 2 h post-MCAO in accordance with the Longa *et al* study (22), and rats with scores of between one and three were held for further experiments. Human TSLP recombinant protein was sourced from Abnova (Taipei, Taiwan). The MCAO rats (n=6/group) were then given either 10 μg of recombinant TSLP or phosphate-buffered saline (PBS) intraperitoneally for 24 h. Subsequently, the rats were sacrificed and cerebral infarct areas were collected.

Cell culture and treatment. HUVECs (American Type Culture Collection, Manassas, VA, USA) were cultured in a RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml streptomycin/penicillin at 37˚C. Cells cultured under normal conditions were maintained in a humidified atmosphere of 95% air and 5% CO_2. Cells in the OGD condition were cultured in the RPMI-1640 without glucose, and maintained in a humidified atmosphere of 94% N_2, 1% O_2 and 5% CO_2 for 2 h. Following this, the OGD-treated cells were cultured in the RPMI-1640 with 5.5 mmol/l glucose under normoxic conditions for reoxygenation for 24 h. The cells subjected to OGD were treated with TSLP at a concentration of 20 ng/ml. LY294002 (50 μM; Cell Signaling Technology, Inc., Danvers, MA, USA) was administered in order to suppress the PI3K/AKT pathway.

ELISA. Tissues of the cerebral infarct area and HUVEC cell supernatants were collected, and the concentration of TSLP was then determined by ELISA assay according to the manufacturer's instructions (DTSLP0, R&D Systems, Inc., Minneapolis, MN, USA).

Western blot analysis. Total proteins were prepared from the cerebral infarct area and HUVEC cells using a Total Protein Extraction kit (Thermo Fisher Scientific, Inc.), and protein concentrations were then determined using a Bicinchoninic Acid protein assay (Pierce; Thermo Fisher Scientific, Inc.). Equal masses of protein samples (50 μg) were subsequently separated on a 10% SDS-PAGE gel and electrophoretically transferred to nitrocellulose membranes. Following blocking with Tris-buffered saline 0.1% Tween (TBST) containing 5% non-fat milk for 2 h at room temperature, the membranes were then incubated with the primary antibodies at 4˚C overnight. The primary antibodies used in these analyses included anti-TSLPR (1:500; sc-517429, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), vascular endothelial growth factor A (VEGFA; 1:800; sc-507, Santa Cruz Biotechnology, Inc.), angiopoietin 2 (Ang-2; 1:400; sc-74402, Santa Cruz Biotechnology, Inc.), phosphorylated AKT (p-AKT; Ser 473 1:400; 12694, Cell Signaling Technology, Inc.), AKT (1:800; 2920, Cell Signaling Technology, Inc.) and GAPDH (1:1,000; sc-47724, Santa Cruz Biotechnology, Inc.). Following washing with TBST, the membranes were further incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000; sc-2005, Santa Cruz Biotechnology, Inc.)

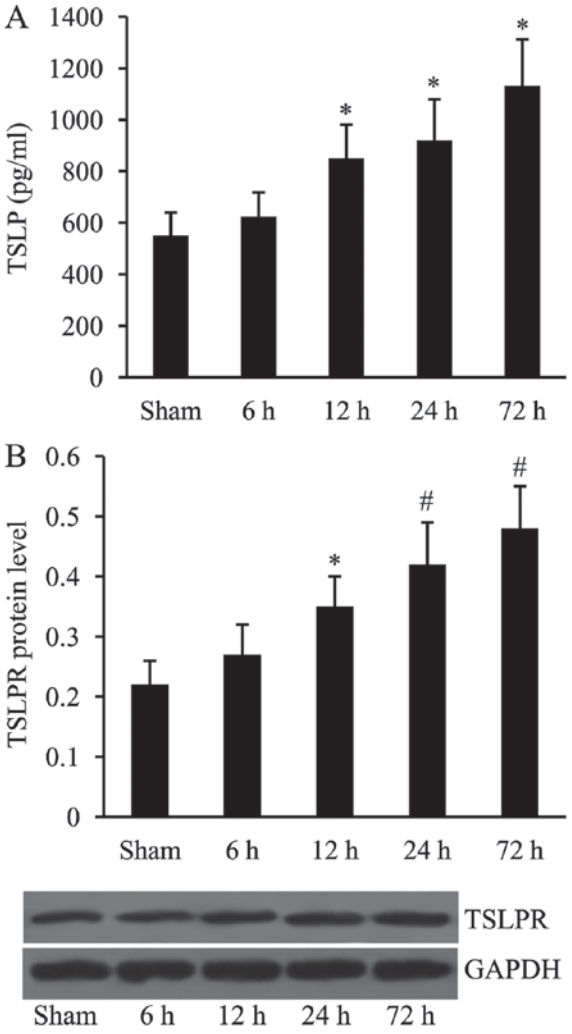

Figure 1. Expression levels of TSLP and TSLPR in the infarct area of rats following MCAO. (A) Expression of TSLP in the infarct area of rats following MCAO and (B) expression of TSLPR in the infarct area of rats following MCAO. n=3. *P<0.05 and #P<0.01 vs. sham group. MCAO, middle cerebral artery occlusion; TSLP, thymic stromal lymphopoietin; TSLPR, thymic stromal lymphopoietin receptor.

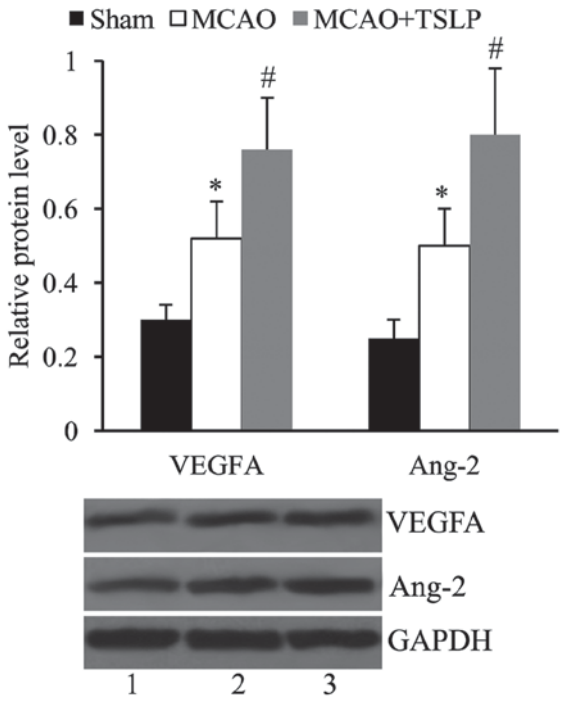

Figure 2. Effect of TSLP (10 μg) on VEGFA and Ang-2 expression in the infarct area of rats following MCAO. Lane 1, Sham; lane 2, MCAO; lane 3, MCAO + TSLP. n=3. *P<0.05 vs. sham; #P<0.05 vs. MCAO. MCAO, middle cerebral artery occlusion; TSLP, thymic stromal lymphopoietin; VEGFA, vascular endothelial growth factor A; Ang-2, angiopoietin 2.

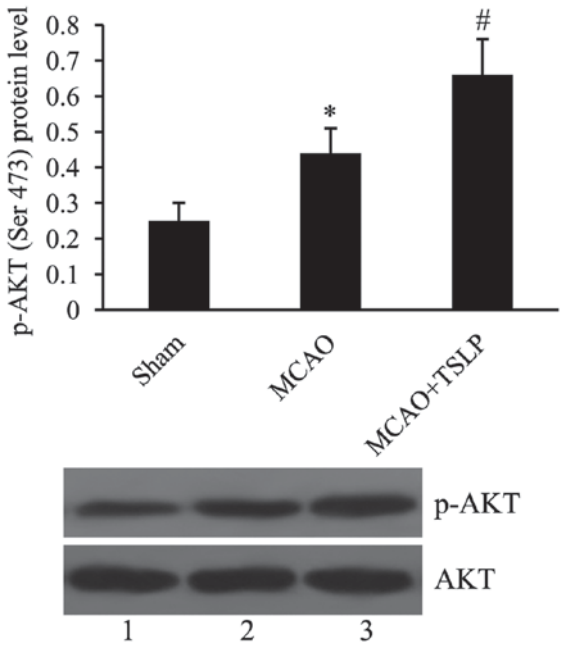

Figure 3. Effect of TSLP on the phosphatidylinositol 3 kinase pathway in the infarct area of rats following MCAO. Akt served as the control. Lane 1, Sham; lane 2, MCAO; lane 3, MCAO + TSLP. n=3. *P<0.05 vs. sham; #P<0.05 vs. MCAO. TSLP, thymic stromal lymphopoietin; MCAO, middle cerebral artery occlusion; AKT, protein kinase B; p-, phosphorylated.

at 37˚C for 2 h. The target bands were then developed using the super ECL reagent (Thermo Fisher Scientific, Inc.). The density of bands was analyzed using Image-Pro Plus, version 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

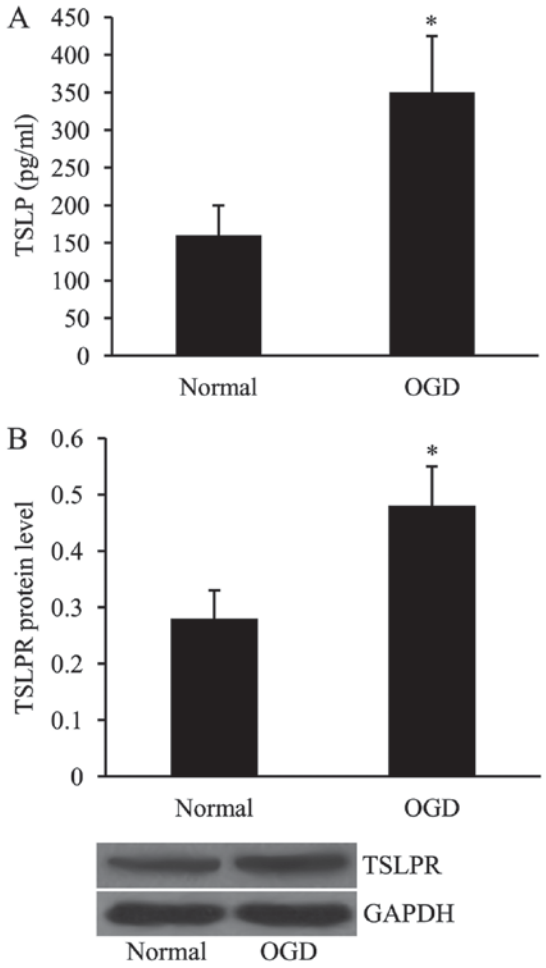

Figure 4. Expression levels of TSLP and TSLPR in HUVECs subjected to OGD. (A) Expression of TSLP in OGD-treated HUVECs and (B) expression of TSLPR in OGD-treated HUVECs. n=3. *P<0.05 vs. normal. HUVECs, human umbilical vein endothelial cells; TSLP, thymic stromal lymphopoietin; OGD, oxygen-glucose deprivation; TSLPR, thymic stromal lymphopoietin receptor.

Cell proliferation assay. The MTT Assay Kit was purchased from Beyotime Institute of Biotechnology (Shanghai, China). According to the manufacturer's instructions, $2x10^3$ cells were plated into the 96-well plates, and treated with 20 ng/ml TSLP. The cells were allowed to grow for 12, 24, 48 and 72 h time intervals, and then 10 μl MTT solution (5 mg/ml) was added into each well. Following incubation at 37˚C for 4 h, 10 μl formazan solution was added into each well and incubated at 37˚C for a further 4 h in order to dissolve the formazan crystals. The absorbance at 570 nm was then determined using a microplate reader (Multiskan Spectrum; Thermo Fishers Scientific, Inc.).

Transwell migration assay. A 6-well Transwell system (8 μm; Corning Incorporated, Corning, NY, USA) was used in the present study to determine cell migration. Following washing with PBS, the cells were suspended in RPMI-1640 cell medium without serum at a density of $5x10^4$ cells/ml in the upper chambers, and 2 ml of cell suspension was then added to the Transwell plates. RPMI-1640 cell medium with 10% fetal calf serum (1 ml) was then added to the lower chambers. The plates were incubated at 37˚C for 24 h, and the upper chamber was

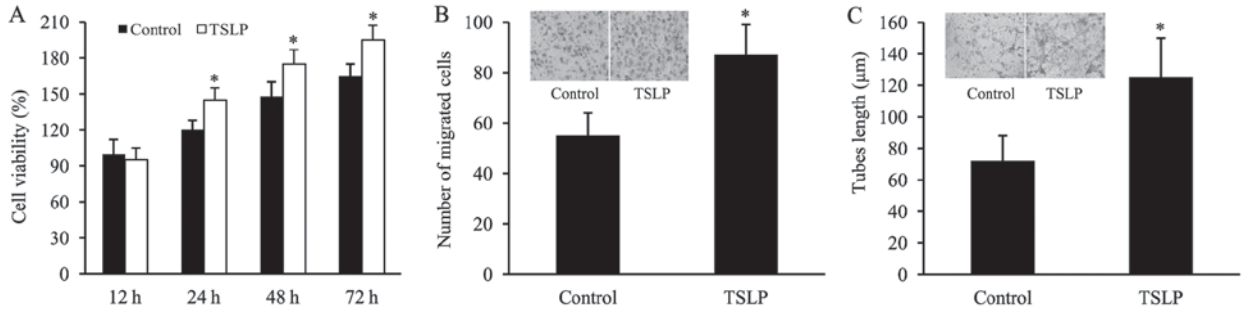

Figure 5. Effect of TSLP on *in vitro* angiogenesis of HUVECs subjected to OGD. Results are displayed as (A) effect of TSLP on cell proliferation of OGD-treated HUVECs, (B) effect of TSLP on cell migration of OGD-treated HUVECs and (C) effect of TSLP on tube length of OGD-treated HUVECs. n=3. *P<0.05 vs. control. HUVECs, human umbilical vein endothelial cells; OGD, oxygen-glucose deprivation; TSLP, thymic stromal lymphopoietin.

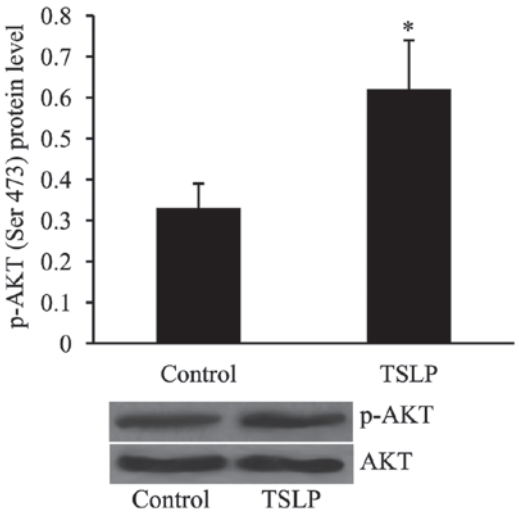

Figure 6. Effect of TSLP (20 ng/ml) on the phosphatidylinositol 3 kinase pathway in human umbilical vein endothelial cells subjected to OGD, AKT served as the control. n=3. *P<0.05 vs. control (cells treated with PBS). TSLP, thymic stromal lymphopoietin; OGD, oxygen-glucose deprivation; AKT, protein kinase B; p-AKT, phosphorylated AKT; TSLP, thymic stromal lymphopoietin.

then fixed in 95% ethanol at room temperature for 15 min and stained with 10% hematoxylin for 15 min at room temperature. The number of migrated cells was revealed using an Eclipse TS100 microscope (magnification x400; Nikon Corporation, Tokyo, Japan).

Tube formation assay. The BD Matrigel matrix (BD Biosciences, Franklin Lakes, NJ, USA) was thawed overnight on ice at 4˚C, and was subsequently added to pre-chilled 24-well plates and incubated at 37˚C for 1 h for solidification. The cells were then digested at a density of $4x10^5$ cells/ml, and 50 μl of the cell suspension was then added to each well. The plates were then incubated at 37˚C, and formation of tube structure was observed 8 h later using a Leica DFC345 FX microscope. Tube lengths for each group were then measured using ImageJ software, version 1.45 (GraphPad Software, Inc., La Jolla, CA, USA), in 10 randomly selected fields.

Statistical analysis. All statistical analyses were performed using SPSS 19.0 (IBM Corp., Armonk, NY, USA), and the data are expressed as the mean ± standard deviation. Comparisons between two groups were performed using the Student's t test, and one-way analysis of variance followed by Fisher's Least Significant Difference test was used to compare the statistically significant differences between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of TSLP and TSLPR is upregulated following MCAO. The rat MCAO model was constructed and the expression of TSLP and TSLPR was then examined using ELISA and western blot analyses at 6, 12, 24 and 72 h time intervals following MCAO. As presented in Fig. 1, compared with the sham at 6 h, the expression levels of both TSLP and TSLPR were significantly increased at 12, 24 and 72 h time intervals following MCAO.

TSLP promotes the expression of VEGFA and Ang-2 following MCAO. In order to investigate whether TSLP affects angiogenesis following MCAO, the rats in the MCAO group were injected with TSLP (10 μg), and the expression levels of VEGFA and Ang-2 in the cerebral infarct area were determined by western blot analysis. It was revealed that compared with the sham, the expression levels of both VEGFA and Ang-2 were significantly upregulated following MCAO. VEGFA and Ang-2 expression levels were further increased in the MCAO rats injected with TSLP (Fig. 2).

TSLP activates PI3K/AKT signaling pathway following MCAO. In order to investigate whether or not the PI3K/AKT pathway could be activated by TSLP following MCAO, p-AKT was investigated using western blot. It was subsequently revealed that the level of p-AKT was significantly increased in the MCAO group compared with the control group. Additionally, 10 μg TSLP injection caused further activation of the PI3K/AKT signaling pathway in the MCAO rats (Fig. 3).

Expression of TSLP and TSLPR is upregulated in HUVECs subjected to OGD. In order to create the *in vitro* MCAO model, the HUVECs were exposed to OGD, and the expression levels of both TSLP and TSLPR were subsequently examined using ELISA and western blot analyses, respectively. As demonstrated in Fig. 4, the expression levels of TSLP and TSLPR in OGD-treated HUVECs, in comparison with the cells cultured under normal conditions, were significantly increased.

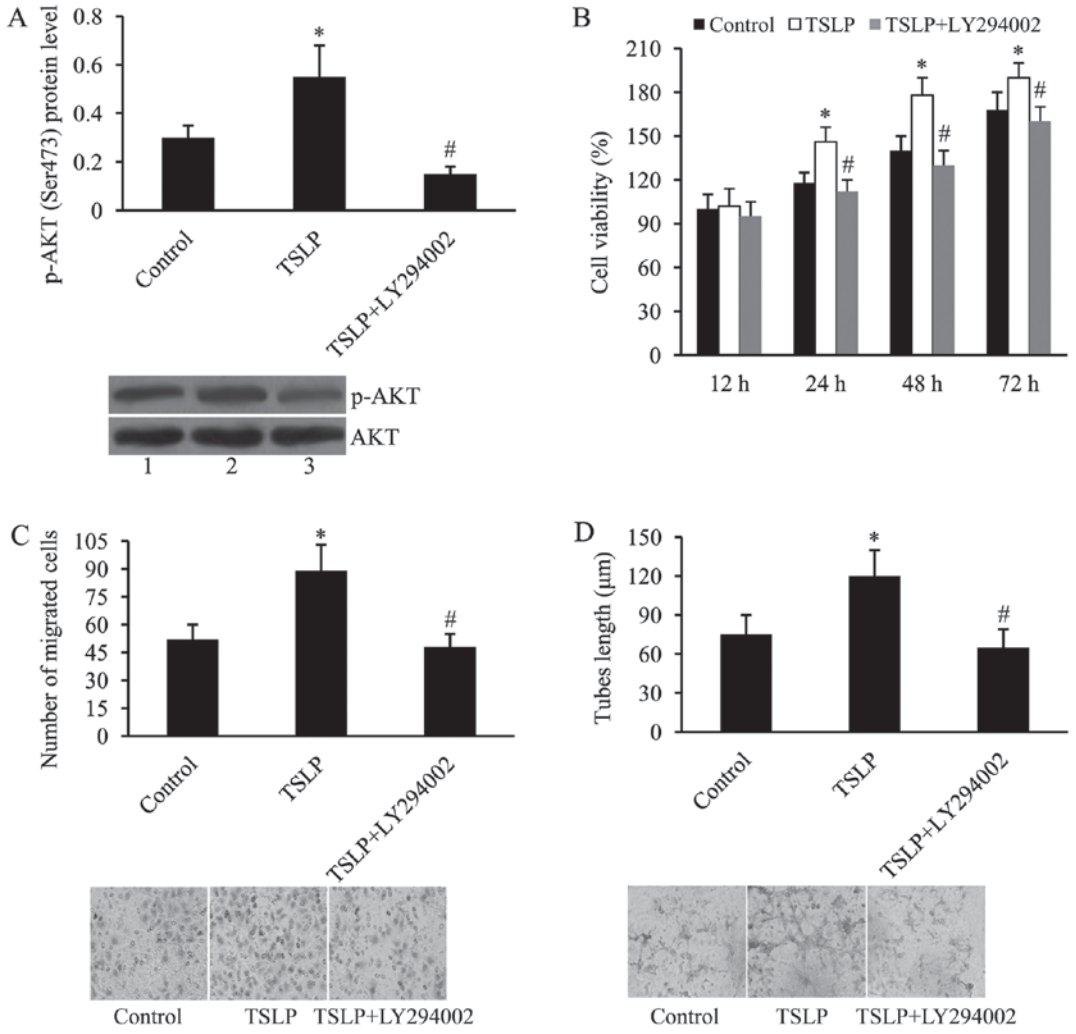

Figure 7. PI3K inhibition attenuates the effect of TSLP on *in vitro* angiogenesis of HUVECs subjected to OGD. (A) LY294002 attenuates TSLP-induced PI3K/AKT activation in OGD-treated HUVECs. AKT served as the control. Lane 1, control; lane 2, TSLP; lane 3, TSLP + LY294002; (B) LY294002 attenuates TSLP-induced cell proliferation of OGD-treated HUVECs; (C) LY294002 attenuates TSLP-induced cell migration of OGD-treated HUVECs; and (D) LY294002 attenuates TSLP-induced tubes length of OGD-treated HUVECs. n=3. *P<0.05 vs. control; #P<0.05 vs. TSLP group. TSLP, thymic stromal lymphopoietin; PI3K, phosphatidylinositol 3 kinase; HUVECs, human umbilical vein endothelial cells; OGD, oxygen-glucose deprivation; AKT, protein kinase B; p-AKT, phosphorylated AKT; LY294002, PI3K inhibitor.

TSLP promotes the in vitro angiogenesis of HUVECs subjected to OGD. The HUVECs subjected to OGD were treated with TSLP, and then cell viability, cell migration and tube formation were examined in order to determine the effect of TSLP on the angiogenic capacity of OGD-treated HUVECs. It was observed that cell viability was significantly increased following TSLP treatment (Fig. 5A). Furthermore, in the TSLP group, the number of migrated cells was significantly increased in comparison with the control (Fig. 5B). Additionally, TSLP treatment led to an increase in the tube lengths of OGD-treated HUVECs compared with the control treatment (Fig. 5C).

TSLP activates PI3K/AKT signaling pathway in HUVECs subjected to OGD. The level of p-AKT was examined in OGD-treated HUVECs in order to determine whether or not the PI3K/AKT pathway is involved in mediating the effect of TSLP on angiogenesis *in vitro*. As revealed in Fig. 6, the level of p-AKT was significantly increased in OGD-treated HUVECs following TSLP treatment compared with the control group.

PI3K inhibition attenuates the effect of TSLP on in vitro angiogenesis of HUVECs subjected to OGD. LY294002, a PI3K inhibitor, was used to determine whether the PI3K/AKT signaling pathway mediates the effect of TSLP on *in vitro* angiogenesis of OGD-treated HUVECs. As demonstrated by western blot analysis, LY294002 inhibited the expression of p-AKT induced by TSLP in OGD-treated HUVECs (Fig. 7A).

Additionally, cell viability was investigated using MTT. It was subsequently revealed that the effect of TSLP on cell proliferation was reversed by the addition of LY294002 (Fig. 7B). In addition, TSLP-induced cell migration was suppressed by LY294002 (Fig. 7C). Tube lengths were also assessed using the tube formation assay. As demonstrated by Fig. 7D, the effect of TSLP on tube formation of OGD-treated HUVECs was attenuated by the addition of LY294002.

Discussion

OGD and MCAO are *in vitro* and *in vivo* cerebral ischemia models (23-26). HUVECs are commonly used as a laboratory

model system for the study of angiogenesis. Previous studies have used OGD-treated HUVECs to investigate stroke (27-29). In this study, an *in vitro* cerebral ischemia model was established by exposing HUVECs to OGD. The present study demonstrated that TSLP/TSLPR promote angiogenesis following ischemic stroke in both animal experiments and cultured cell experiments. Furthermore, it was confirmed that TSLP/TSLPR may exert effects on angiogenesis via the PI3K/AKT signaling pathway.

Several studies have previously reported that TSLP is involved in the pathogenesis of atherosclerosis, diabetes, obesity and asthma (30-32). Recently, Kitic *et al* (33) reported that TSLP is also expressed in the central nervous system, and that microglial cells express TSLPR. The expression of TSLP in the central nervous system varies among different pathological conditions (33). However, whether TSLP/TSLPR are involved in the pathogenesis of ischemic stroke remains unknown. In the present study, a rat MCAO model was established, and it was revealed that the expression levels of TSLP and TSLPR were significantly increased in the infarct area between 12 and 72 h following MCAO. An *in vitro* MCAO model was constructed by exposing the HUVECs to OGD, and it was revealed that the expression levels of TSLP and TSLPR were significantly increased in HUVECs subjected to OGD compared with those cultured under normal conditions. These results suggest that TSLP/TSLPR may be involved in the pathogenesis of ischemic stroke.

Angiogenesis is a key neurorestorative event in response to ischemia (8). Following ischemic stroke, angiogenesis occurs in the ischemic boundary zone and improves neurological function (34,35). VEGFA is an essential molecule in both physiological and pathological angiogenesis. This growth factor induces proliferation, differentiation and migration of vascular endothelial cells. Following ischemic stroke, elevated levels of VEGFA promote capillary formation and increase blood flow to the area surrounding the infarction (36). Ang-2 may facilitate endothelial cell migration and proliferation in co-ordination with VEGFA, thus acting as an angiogenic signal (37). A previous study reported that TSLP stimulates the proliferation and activation of HUVECs, and upregulates the expression of angiogenesis-associated molecules, CD62E and CD105 (21). Consistent with these findings, the *in vitro* results of the present study demonstrated that TSLP treatment promoted cell proliferation and migration, and induced tube formation of OGD-treated HUVECs. In addition, the *in vivo* results revealed that the expression levels of angiogenic molecules, VEGFA and Ang-2 were increased following MCAO. TSLP injection further upregulated VEGFA and Ang-2 expression in the infarct area. These results suggest that TSLP promotes angiogenesis following ischemic stroke in both *in vivo* and *in vitro* conditions.

The PI3K/AKT signaling pathway participates in various cellular activities, such as cell proliferation, apoptosis, differentiation and inflammatory responses. Additionally, the activation of the PI3K/AKT signaling pathway has been revealed to be implicated in the occurrence and development of angiogenesis (38,39). Previous evidence has demonstrated that TSLP/TSLPR functions via activation of the PI3K/AKT pathway in order to induce platelet activation (40). In the present study, it was investigated whether the PI3K/AKT signaling pathway mediates the effects of TSLP/TSLPR on angiogenesis following ischemic stroke. Consistent with the aforementioned study (40), TSLP was demonstrated to activate the PI3K/AKT signaling pathway in OGD-treated HUVECs. LY294002, a PI3K inhibitor, was used in this study to suppress the PI3K/AKT signaling pathway. Subsequently, the effects of TSLP on *in vitro* angiogenesis of OGD-treated HUVECs were attenuated by LY294002.

In conclusion, the findings of this study suggest a novel mechanism underlying the role of TSLP/TSLPR in angiogenesis following ischemic stroke. This study, to the best of our knowledge, is the first to demonstrate that TSLP/TSLPR promote angiogenesis following ischemic stroke *in vivo* and *in vitro*, and that these effects are mediated, at least partially, via the activation of the PI3K/AKT signaling pathway. Therefore, TSLP/TSLPR may be potential therapeutic targets for ischemic stroke treatment. Further studies are required to confirm the effects of TSLP/TSLPR on angiogenesis in clinical stroke patients, examine the expression of TSLP/TSLPR in brain tissues and investigate angiogenesis using imaging tests in infarct areas following TSLP/TSLPR treatment.

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